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ATROPINE AND RELATED COMPOUNDS ON THE METABOLISM OF ELECTRICALLY STIMULATED SECTIONS OF MAMMALIAN CEREBRAL CORTEX

BY

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(Received April 30, 1951)

Several substances which act on the central nervous system produce large effects on the metabolism of the brain during their action *in vivo*, but have little effect on the same metabolic processes when these are studied in preparations from the brain *in vitro*. In recent studies, narcotics and convulsants were shown to behave in this way towards the respiration and lactic acid formation of mammalian brain. It was concluded from this that the effects of the drugs on metabolism *in vivo* were secondary to effects which they had on functional activities of the brain, such as the transmission of impulses, and were thus likely to be shown only in tissue in a comparable state of activity (Buchel and McIlwain, 1950 ; Anguiano and McIlwain, 1951).

Means have now been developed (McIlwain, 1951a) of stimulating portions of separated mammalian cerebral cortex electrically, under good metabolic conditions. After stimulation, the levels of respiration, glycolysis, inorganic phosphate, and phosphocreatine of the tissue all changed in the sense in which they change during increased activity of the brain *in vivo*. These methods should therefore be applicable to the study of agents such as the narcotics and convulsants. The first necessity, however, was to examine further the extent to which the increased metabolism was related to increased activity of a functional character, e.g., to spread of impulses. For this reason we have examined first the actions of agents which might block such spread, and report in the present paper the effects of a group of substances related to acetylcholine. The metabolic processes examined have been respiration and lactic acid formation. These are the main energy-yielding reactions in nervous tissues.

EXPERIMENTAL

Tissue metabolism.—Details are given elsewhere (McIlwain and Grinyer, 1950 ; McIlwain, Buchel, and Cheshire, 1951). The brain was removed from rats and guinea-pigs, the cortex sliced, and the slices examined in aerated, glucose-containing salines. Respiration was determined manometrically by three- or five-minute readings, which enabled its course to be followed closely. Lactic acid was estimated in the saline at the end of the experiments. Also, in a few instances, the course of its formation was followed by manometric measurements of the CO_2 evolution from bicarbonate salines. For electrical stimulation of slices, tissues were fixed in electrodes D in vessels " A "

of McIlwain (1951a), where full experimental details are given. Substances to be added during an experiment were placed first in the sidearm of a manometric vessel.

Chopped tissue and slice-fragments were examined while suspended in nutrient salines in the electrode vessels D and E described by McIlwain (1951b), where details of the preparation of the tissue are also given.

Stimulation.—Alternating current was of 50 cycles per second and at the root mean square voltages which are indicated. Condenser pulses were diphasic, from a circuit (McIlwain, 1951a) in which a condenser was alternately charged and discharged from a battery, through the experimental vessel, with a pulse frequency of 100/sec. unless indicated otherwise. Duration and peak voltage of the pulses were measured with an oscilloscope; the duration given is the time-constant (msec. required for the pulse to fall to 1/3 of its peak value).

RESULTS

Atropine on normal and electrically stimulated metabolism

On respiration.—Atropine, when added in concentrations up to 10^{-3} M to media in which normal unstimulated brain slices were respiring, was without effect on their rate of oxygen uptake. Table I and parts of Fig. 1 show this. In Fig. 1, rates are given by the slopes of the lines; line A, *a* (without atropine) is of very similar slope to lines A, *b*; B, *d*; and C, *a* and *c* (with atropine).

In marked contrast to this was the effect of atropine on the respiration of stimulated slices. The extent of the electrical stimulation in the absence of added substances can be seen by comparing curves A and D (Fig. 1) or the initial part of curves

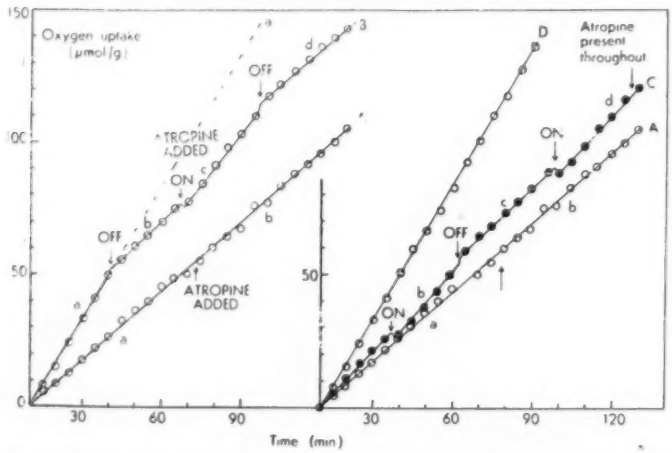

FIG. 1.—Atropine (final concentration 8×10^{-4} M) on respiratory response to stimulation. Cerebral cortex slices were fixed to electrodes in vessels containing glucose-phosphate saline saturated with O_2; CO_2 absorbed in NaOH-paper. Oxygen uptake was calculated from 5-min. pressure readings. Stimulation was by condenser pulses of 21–23V and time-constant 0.5 msec., applied as indicated. (A): unstimulated; atropine added at 66 min. (B): *a* and *c*, stimulated; atropine added at 66 min. (C) (closed circles): atropine present throughout; stimulated at *b* and *d*. (D): stimulated throughout.

A and B. The stimulation shown in the first part of B has been shown in other experiments to be maintained for long periods as indicated by D or the dotted line *a*. Table I shows that a typical increase on stimulation by the impulses used

was to 200 per cent of the normal rate. Condenser pulses of the characters quoted gave stimulation approaching the maximum of which the tissue was capable, with minimum applied voltage. Lesser voltage gave markedly less stimulation (Table I). The electrical energy which is applied in stimulating the tissue causes a very small rise in temperature of the experimental vessels (about 0.02° C.), which is seen in Fig. 1 as a small displacement of the lines at the points at which current has been started and stopped.

Stimulation in the presence of atropine gave a much smaller increase in respiratory rate. The contrast in Fig. 1 between lines D (without atropine) and C, *b* and *d* (with atropine) shows this. The action of atropine comes into effect quickly ; the substance was added in B, *c* (Fig. 1) at the same time as stimulation was begun, and the stimulation is seen to be much less effective than in B, *a*. Moreover, the effect persisted for at least 1 to 2 hours after addition of the drug (C, *b* and *d*, Fig. 1).

TABLE I

ATROPINE AND RELATED COMPOUNDS ON RESPIRATION OF GUINEA-PIG CEREBRAL CORTEX

The experimental arrangement was similar to that of Fig. 1 ; the rates quoted below are the slopes of lines such as those shown in the Fig. As the action of atropine was found to be prompt and persistent, the experimental period during which the rates were measured has not been recorded ; it varied between ½ and 2 hr. after the commencement of the experiment. Stimulation was always by condenser pulses, which were at the potential quoted and which were found by measurement on an oscilloscope to be of time constant 0.5–0.7 msec.

Preparation	Applied potential (V)	Drug added (M)	Rate of respiration (μmol./g./hr.)			
			Unstimulated		Stimulated	
			No drug	With drug	No drug	With drug
Slices in electrodes	24	Atropine 8×10^{-4}	60, 58, 60, 66, 61	62, 56, 60, 62, 61	130, 140, 124, 126 127	74, 82, 74, 81, 73
Slices in electrodes	24	Atropine 10^{-4}	60, 66, 61	61, 66, 63	124, 126, 127	124, 123, 120
Slices in electrodes	24	Atropine 10^{-5}	60	63	124	130
Slices in electrodes	12	Atropine 10^{-3}	61	61	98	61
Slices in electrodes	12	Atropine 10^{-4}	68	63	98	95
Slice-fragments, free	24	Atropine 8×10^{-4}	67	65	150	80
Chopped, free ..	24	Atropine 10^{-3}	68	64	109	68
Slices in electrodes	24	Hyoscine 10^{-3}	66	58	107	74
Slices in electrodes	24	Hyoscine 10^{-4}	72	69	127	109
Slices in electrodes	24	Hyoscyamine 10^{-3}	64	58	120	68
Slice-fragments ..	24	Cocaine 2×10^{-3}	63	61	125	66
Slice-fragments ..	24	Cocaine 2×10^{-4}	63	62	125	69

Several experiments are summarized in Table I in which the concentration of atropine, the preparation of the cortex, the stimulating potential, and the substances added were varied. The effective concentrations of atropine were found to be between 1 and 8×10^{-4} M (total concentration of both optical isomerides). Concentrations of 10^{-4} M and below had little effect, even with a relatively weak stimulus (12V) which gave an approximately 50 per cent increase in respiratory rate.

No marked difference was found when the effects of atropine on slices were compared with those on slice-fragments or on chopped cortex. Atropine has also

been found to act on some other parts of the brain (unpublished), but accurate comparisons have not yet been made. Throughout, the effects of the drugs on unstimulated respiration of the different preparations has been very small.

On glycolysis.—The stimuli effective in increasing respiration, without added drugs, have an even greater effect on the formation of lactic acid from glucose. Table II includes experiments in which the increase has been to over 300 per cent

TABLE II

ATROPINE AND COCAINE ON LACTIC ACID FORMATION

Slices of guinea-pig cerebral cortex were fixed to electrodes in a series of vessels containing glucose-phosphate saline saturated with O_2. Atropine, where indicated, was present throughout the experiment. Stimulation was by condenser pulses of 21–23V and 0.5–0.6 msec., applied to vessels with and without atropine; others were unstimulated. At the end of the experimental period, portions of the salines were taken for determination of lactic acid

Substance added (M)	Duration (min.) of		Lactic acid formation (μmol./exp.)			
	Whole exp.	Stimulation	Unstimulated		Stimulated	
			No drug	With drug	No drug	With drug
Atropine 8×10^{-4} ..	85	48	49	60	109	75
Atropine 10^{-4}, 10^{-5} ..	85	48	49	52	109	107, 109
Atropine 8×10^{-4} ..	92	50	34	38	109	58
Atropine 10^{-4} ..	92	50	34	34	107	85
Atropine 8×10^{-4} ..	125	90	31	—	87	39
Atropine 10^{-4} ..	125	90	31	—	87	78
Cocaine 2×10^{-4} ..	130	90	42	47	88	53
Cocaine 2×10^{-3} ..	130	90	42	55	88	65

of the initial rate. In calculating the percentage increase, use is made of the knowledge that the increased rate is regular, and that it begins promptly on starting the stimulus and ends promptly when stimulation is stopped. The active concentration of atropine was again between 10^{-3} and 10^{-4} M. Thus the mean stimulation without added substances, in the experiments of Table II, was to 390 per cent of the normal rate. The mean value in the presence of 10^{-4} M atropine was to 332 per cent, and with 8×10^{-4} M to 186 per cent. Control experiments showed these concentrations of atropine to have very little action on the normal glycolysis of unstimulated slices; lactic acid in the presence of 10^{-3} M atropine was found to be 106, 95, and 88 per cent and with 10^{-4} M 112 and 92 per cent of normal slices.

Manometric experiments showed the action of atropine to be prompt. These experiments, of which Fig. 2 gives examples, depended on the pressure changes observed in bicarbonate salines in equilibrium with O_2–CO_2 mixtures. Lactic acid displaces CO_2 from the saline, leading to increase in pressure, which is measured. Pressure is also affected by respiration, which absorbs O_2 and forms CO_2, but, as the respiratory quotient of brain cortex is nearly unity and remains so on stimulation, the respiratory exchange leads only to a small fall in pressure due to the greater solubility of CO_2 than O_2 in the saline. The pressure changes have been calculated as μmol. CO_2/g., which can be taken as approximately equivalent to the lactic acid formed per g. tissue; the measure is not exact, but has been shown

(McIlwain, Anguiano, and Cheshire, 1951) to be applicable to experiments of the present type.

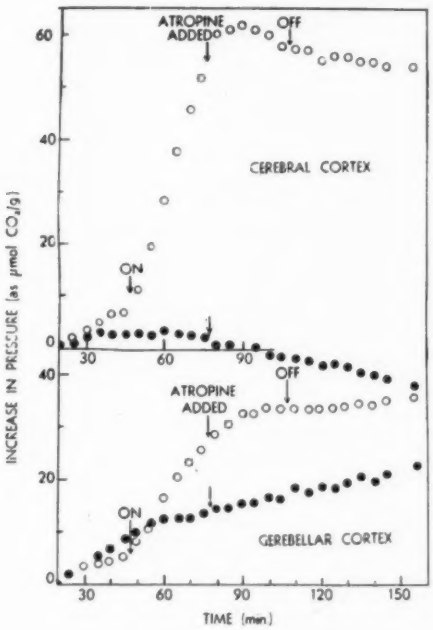

FIG. 2.—Atropine on glycolysis. Fragments of the tissues (average wt., 2 mg.) were floating freely in bicarbonate-glucose salines in equilibrium with O_2–5% CO_2. Open circles with both cerebral and cerebellar cortex: stimulation as described in Fig. 1 applied between 47 and 107 min.; closed circles: unstimulated. Atropine (to 8×10^{-4} M) added to all at 76–77 min.

In Fig. 2, atropine will be seen to have no effect on the course of acid formation by either cerebral or cerebellar cortex in the absence of stimulation. Stimulation greatly increased the rate of acid formation ; other experiments showed this increase to persist for over an hour. It was, however, promptly antagonized by atropine. The effect of atropine was seen within five minutes of its addition and became maximal within fifteen minutes. By this time the pressure change was becoming parallel in course to that of the unstimulated tissue, and little change took place on switching off the source of impulses.

Atropine on tissue stimulated by other means

The respiration and glycolysis of slices of cerebral cortex can be increased not only by electrical stimulation but also by the addition of certain substances to media in which they are metabolizing. In order to assess the relation between atropine and electrical stimulation, the effect of atropine on slices stimulated by these other means was examined. Experiments with 2 : 4-dinitrophenol and with high concentrations of potassium salts are shown in Table III. Potassium chloride was used at concentrations which gave a stimulation as great as that obtained electrically, and also at lower concentration giving a submaximal stimulation and presumably more scope for an antagonist. No antagonism was, however, shown by atropine to the action of KCl, when atropine was used at concentrations up to 10^{-3} M. These concentrations will be seen from previous Tables, and also from the parallel experiment of Table III, to have major effects in antagonizing the metabolic

results of electrical stimulation. The lack of antagonism to KCl extended to both respiration and glycolysis. Findings with 2 : 4-dinitrophenol were similar (Table III).

TABLE III

ATROPINE ON STIMULATION CAUSED BY VARIOUS AGENTS

Slices of guinea-pig cerebral cortex were used in glucose phosphate saline in a series of vessels. Atropine was present throughout in half of these, and the stimulating agents were added during the experiment; respiration was measured throughout and lactic acid determined at the end of the experiment. Electrical stimulation was by condenser pulses of 24V, and 0.5 μsec. duration

Stimulating agent	Respiration (μmol.O_2/g./hr.)				Lactic acid (μmol./g./exp.)		
	With no addition	With stimulating agent	With 10^{-3} M atropine	With stimulating agent and 10^{-3} M atropine	No addition	With stimulating agent	With stimulating agent and 10^{-3} M atropine
0.088 M-KCl ..	60	128	58	132	35	60	61
0.029 M-KCl ..	60	98	58	104	—	—	—
2×10^{-5} M-dinitrophenol ..	62	118	64	127	—	—	—
5×10^{-6} M-dinitrophenol ..	62	123	64	135	33	40	48
Electrical ..	62	117	64	66	33	66	31

Hyoscine, hyoscyamine, and cocaine

The concentrations of atropine active in the preceding experiments are relatively high (see Discussion), and information on their nature has been sought by examining some related compounds. Table I shows that, as would be expected, (-)-hyoscyamine was also active. (-)-Hyoscine was possibly slightly more active than atropine. Moreover, cocaine was as effective as atropine and acted in somewhat lower concentrations. The effect of cocaine extended to glycolysis also (Table II).

Eserine and acetylcholine

Eserine, in concentrations between 10^{-6} and 10^{-4} M, had no major effect on the respiration or glycolysis of unstimulated cerebral cortex (Table IV). Its effect on stimulated tissue was examined under conditions which gave a response approaching maximal, and also using stimuli which gave much smaller responses. Again, any effects were small. No consistent increase or decrease of the stimulation was observed, and atropine had its normal depressant effect in the presence of eserine. Submaximal stimuli were examined to see whether atropine caused any potentiation of the effect of electrical stimulation. The values of Table IV show small increases in some experiments but not in others, and in some experiments small comparable increases are shown in the absence of stimulation. None of these are considered significant.

In addition to the results quoted in Table III, experiments were run in bicarbonate saline and the course of excess acid formation followed manometrically. Again, no potentiation of submaximal stimuli was seen.

TABLE IV

ESERINE ON RESPIRATION AND LACTIC ACID FORMATION

Experiments used slices from the cerebral cortex of guinea-pigs, except with the last experiment, which was with rat tissue; they were with phosphate-glucose saline except where indicated. Stimulation was by condenser pulses at 100/sec. of the voltage and duration (time to fall to 1/3 of peak voltage) quoted. Vessels were unstimulated for two periods during the experiment, to obtain normal unstimulated rates of respiration; these were between 63 and 70 μmol./g./hr. The lactic acid formation refers to the whole experimental period

Preparation	Agent (M)	Stimulation		Respiration (% of normal controls)		
		V	Time-constant (μsec.)	Stimulated without drug	Stimulated with drug	Unstimulated with drug
Slices in electrodes	Eserine 10^{-4}	24	0.6	178	195	102
Slices in electrodes	Eserine 10^{-4}	24	0.6	170	164	94
Slices in electrodes	Eserine 10^{-4} + Atropine 10^{-3}	23	0.6	228	112	95
Slice-fragments, free	Eserine 10^{-4}	12	0.5	146	154	97
Slice-fragments, free	Eserine 10^{-4}	28	0.1	134	132	97
				Lactic acid formation (% of normal control)		
Slice-fragments, free	Eserine 10^{-4}	12 28	0.5 0.1	183	204	117
Slice-fragments, free (in bicarbonate saline)	Eserine 6×10^{-5} 6×10^{-5}	8 20	0.5 0.1	172	186	103

The effect of acetylcholine itself, in the presence or absence of eserine, was also examined. In these experiments, acetylcholine concentrations between 10^{-4} and 10^{-6} M were used, and these were sometimes present with eserine from the beginning of the experiment, and at other times added during the experiment. No stimulation (or depression) was caused by the acetylcholine, in respiration or glycolysis.

Discussion

The increased metabolic activity on stimulating electrically separated tissues of the central nervous system has only recently been observed (McIlwain, 1951a), and many aspects of its nature remain to be characterized. The present results contribute to this characterization. It is already known (McIlwain, 1951a, b, and unpublished exps.) that the stimulation *in vitro* is similar to that occurring during increased activity of the central nervous system *in vivo*, with respect to: (i) the nature and magnitude of the changes which take place in respiration, glycolysis, and in inorganic and creatine phosphates; (ii) the frequency, duration, and potential of the exciting impulses; (iii) the promptness and persistence of the responses. The present observations contribute to a fourth group of characteristics, namely those relating to added substances.

Atropine has been valuable in the present studies in giving a very clear example of a substance without effect on normal levels of respiration and glycolysis, but with a major effect on the increased respiration and glycolysis which result from electrical stimulation. Moreover, atropine is without effect on the increase in

respiration and glycolysis which is brought about by 2 : 4-dinitrophenol or by KCl. In magnitude, the effects of these agents are similar to that of electrical stimulation. A relatively specific relation of atropine to stimulation by electrical means is thus suggested.

Antagonism to electrical stimulation was found not only with atropine but also with hyoscine and cocaine. This action of cocaine may be compared to its action in blocking the passage of impulses in electrically stimulated peripheral nerves on local application (see, for example, Kato, 1936 ; Schaefer, 1942). The concentrations of atropine needed ($1 - 8 \times 10^{-4}$ M of the racemic compound) are much above those acting peripherally, and also are probably above those at which any specific central effects of atropine are observed after its general administration. It is doubtful whether even the 200 mg./day administered orally in some cases of Parkinsonism (Hall, 1937 ; Cohen and Craw, 1937), or the doses of up to 1 g. orally found to produce psychoses (see, for example, Alexander, 1946), would give concentrations of 10^{-4} M (29 mg./l.) in tissue fluids, though such values afford only an approximate basis for comparison. However, the concentrations found effective in the present experiments are certainly lower than those of 200 mg./l. (applied directly to the exposed optic lobes) found by Chauchard (1939–40) to alter the electrical response to stimulation of frog brain, and may be compared to the toxic intravenous dose of some 50 mg./kg. which shows central actions in dogs or cats (Koppanyi, 1939). Also, concentrations which are high in relation to those active *in vivo* may be necessary in our experiments on account of our means of stimulation or of the tissue chosen for the present work.

The present findings with atropine do not offer any unambiguous evidence with respect to the participation of cholinergic systems in electrical stimulation of the cortex. Findings with eserine and acetylcholine were also negative. With eserine, a range of concentrations was used, and submaximal electrical stimuli were applied in order to see possible augmentation of their effects, but none was found. As our findings are not exhaustive, they do not at all contradict the considerable evidence for cholinergic systems in the brain as a whole. Such systems appear much less important in some parts of the brain (Feldberg, 1945). Our observations do, however, illustrate properties of whatever systems are brought into action by the stimulation *in vitro*. In considering the neuronal elements which might be involved, observations on stimulation and recording at the cerebral cortex *in vivo* are also relevant. Spread of activity following such stimuli appears to involve dendritic connexions of cortical cells (Adrian, 1936 ; Chang, 1951 ; see also Burns, 1950) and can be inhibited by procaine. The sensitivity of the metabolic stimulation to other agents is being studied.

Summary

1. The increase in respiration and glycolysis of separated portions of cerebral cortex brought about *in vitro* by electrical stimulation was inhibited by atropine and by some structurally related compounds.

2. Atropine, hyoscine, hyoscyamine, and cocaine were effective in concentrations of about 10^{-4} M. When glycolysis had been increased to 300–400 per cent of its original rate, and respiration to about 200 per cent, these substances could reduce the processes to 100–150 per cent of their original rates.

3. The concentrations of the added substances which were effective as antagonists to the electrically stimulated metabolism had little or no action on normal respiration and glycolysis of the tissue.

4. Atropine was also ineffective in antagonizing similar increases in respiration and glycolysis brought about by potassium salts or by 2 : 4-dinitrophenol.

5. Eserine was without action on the normal or stimulated metabolism of the present preparations and acetylcholine with or without eserine did not produce the effects of electrical stimulation.

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THE ACTION OF *d*-TUBOCURARINE ON THE MONOSYNAPTIC EXTENSOR REFLEX

BY

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It has been believed for nearly 150 years that curare has a stimulant action on the central nervous system in addition to its well-known peripheral effect on neuromuscular transmission. The evidence was conflicting until Tillie (1890) published the results of a systematic investigation of the effects of curare on the central nervous system of the frog. He showed that much of the previous confusion had been due to the unpredictable action of curare on the circulation. By means of intra-aortic injections and local application of curare to the spinal cord he was able to demonstrate a strong stimulant effect on the central nervous system. This effect often amounted to tetanus and was present in both parts of the spinal cord after subdivision. He further showed that the depression of reflex activity often seen in the intact frog after the subcutaneous injection of curare was due to an inhibitory influence by the higher centres. He also demonstrated that the " strychnine-like " action of curare was not due to contamination by strychnine.

McGuigan (1916) studied the effect of the local application of curare to the central nervous system in dogs. He confirmed the excitatory action of curare, but emphasized that the general thrashing movements observed were quite unlike the spasms produced by strychnine. This stimulating action was confirmed by numerous other workers, including Joseph and Meltzer (1911), Santesson (1920–1), Blume (1934), West (1937), and Euler and Wahlund (1941). The literature is fully discussed by McIntyre (1947).

Cohnberg (1946) studied the action of curare administered by subcutaneous, intravenous, intramuscular, and intraperitoneal injection into intact animals of various species. He observed reflex hyperexcitability and convulsions in all the species examined (rat, cat, and rabbit). He showed that asphyxia was not responsible, and concluded that curare produces convulsions in mammals by stimulation at a higher level of the C.N.S. than the spinal cord, in contrast to strychnine. Eccles (1946), however, applied curare to the isolated frog spinal cord and found that large doses set up sustained spontaneous convulsant activity which resembled the action of strychnine.

McCawley (1949) found that small doses of *d*-tubocurarine administered intravenously to cats produced muscular twitchings, convulsions, and changes in the electroencephalogram, and he showed that these effects could be prevented by the

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previous administration of pentobarbital, thiopentone, or dial-urethane. This author investigated the effects of *d*-tubocurarine on the monosynaptic reflexes in cats anaesthetized with pentobarbital and found only a depression of amplitude. Salama and Wright (1950) administered *d*-tubocurarine chloride by intraventricular, intracisternal, and intrathecal injection to chloralosed, decerebrate, and high spinal cats. They showed that small doses produce heightened reflex excitability of the spinal cord and later generalized convulsions. They concluded that "the effects on the spinal cord are due only to a minor extent to a direct action on the spinal centres: they are mainly the result of stimulation of the cells of origin of the facilitatory pathways in the brain."

Naess (1950), however, could not find any effect on the mono- and poly-synaptic reflexes in the cat after the administration of *d*-tubocurarine intravenously. In these experiments the cats were anaesthetized with dial.

Baisset, Laporte, and Grezes-Rueff (1949) report that *d*-tubocurarine, injected intravenously, gives a transient increase followed by a disappearance of the monosynaptic reflex in the decapitated unanaesthetized dog only when a rapid injection of curare is followed by a drop of the blood pressure from 40 to 25 mm. mercury.

In the course of other studies on the effect of *d*-tubocurarine on excitability changes in the spinal cord, we noted that the drug had a marked effect on the monosynaptic reflex response. This reflex appeared to be a sensitive indicator of part of the central effect of tubocurarine on the nervous system, and its action was studied in various types of preparation. The effect of *d*-tubocurarine on the polysynaptic reflex as well as a comparative study on the effect of strychnine is reported in another paper (Bernhard, Taverner, and Widén, 1951).

Methods

Cats were used in all experiments. The preliminary decerebration or decapitation was performed under ether anaesthesia. The experimental procedures were begun more than two hours later so that the effect of the ether had disappeared. After the preliminary preparation lumbar laminectomy was performed and the appropriate roots were prepared intradurally. Usually the L_6, L_7, and S_1 ventral roots were cut distally and the L_7 or S_1 root was placed on the recording electrodes. The nerves to the two heads of the gastrocnemius muscle were dissected, cut distally, and one branch was placed on the stimulating electrodes. All exposed nervous structures were covered with paraffin oil. The electrical stimuli were applied through a transformer. Small supraliminal test responses were used throughout. The ventral root responses were amplified by a differential amplifier and recorded photographically from a cathode ray oscillograph. The curare preparation used was *d*-tubocurarine, 20 U (3 mg.)/c.c. (Abbott). All the injections were given slowly into a vein of the forelimb.

The procedure adopted was to set up the preparation and elicit a monosynaptic reflex response from one of the nerves to the gastrocnemius by electrical stimulation at intervals, usually of 2 seconds. When the response had settled to a fairly constant amplitude control records were taken for periods of 0.5–1 minute at a stimulus frequency of 30/min. The *d*-tubocurarine was then injected intravenously and sample records were taken for periods of approximately 30 seconds, at intervals of 1–2 minutes, for a total period of 10–20 minutes. The oscillograph screen was observed throughout, and any special events were recorded as required. The height of the monosynaptic reflex response was measured without magnification. In the decerebrate preparations artificial respiration was begun as soon as any evidence of respiratory failure appeared. All

other preparations received artificial respiration from an electrically driven pump throughout. In a series of experiments the blood pressure was recorded simultaneously from the carotid artery.

The effect of the experimental procedures upon the amplitude of the monosynaptic reflex response was expressed graphically in terms of the average pre-injection test response. Each point was obtained from the mean of the amplitude of five successive responses at 2-second intervals expressed as a percentage of the pre-injection response amplitude. In graphs which show the effect of repeated injections the stimulus strength remained constant throughout and the preparation was not disturbed. If necessary the amplifier gain was reduced, allowance being made for this in computing the results. The illustrations show frames, selected from the film records of the experiments, chosen to give a representative picture of the events under consideration.

Results

Threshold dose of tubocurarine

Stimulation of the afferent fibres with lowest threshold in the gastrocnemius nerve is followed by a well-synchronized reflex potential in the ventral root. Fig. 1 A shows such a potential. The time marker represents intervals of 1 msec., and the interval between the shock artifact and the initiation of the reflex spike is 2.5 msec. It has been shown by Lloyd (1943) that in such a preparation afferent conduction to the spinal cord requires approximately 1.4 msec. and in the ventral root about 0.3 msec. By subtraction approximately 0.8 msec. is available for synaptic delay and central conduction. This short time interval suggests strongly that two-neurone arcs are involved (Lorente de Nó, 1935 ; Renshaw, 1940), i.e., monosynaptic reflex arcs. The same author has shown that this discharge (Group I reflex) reflects into the muscle, the large afferent fibres of which are stimulated (Lloyd, 1943), and therefore the reflex spike may be regarded as representing activity in the extensor motorneurones.

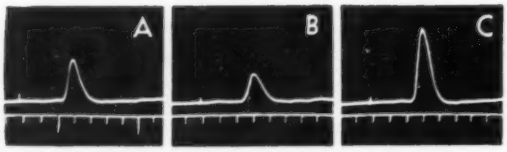

FIG. 1.—Reflex discharge (monosynaptic response) recorded in L_7 ventral root after stimulation of the ipsilateral gastrocnemius nerve (A) before, (B) 45 sec. and (C) 5 min. after, intravenous injection of 0.11 mg. tubocurarine per kg. Time in msec.

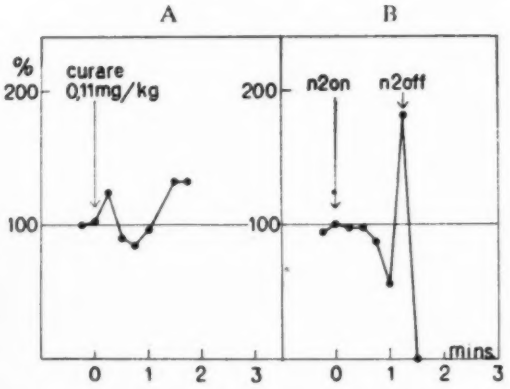

FIG. 2.—(A) Effect of 0.11 mg. tubocurarine per kg. on the monosynaptic response in a decerebrate cat. The amplitude of the monosynaptic spike in per cent of pre-injection test response; (B) Effect of asphyxia (N_2 on—N_2 off) on the amplitude of the monosynaptic reflex discharge.

In Fig. 1, B and C show the changes in amplitude of the monosynaptic response 45 seconds and 5 minutes respectively after the intravenous injection of 0.11 mg. tubocurarine per kg. body weight into a decerebrate cat. Fig. 2 A is a graph showing the changes in amplitude of the monosynaptic response plotted as a percentage of the pre-injection test response. There is a slight increase in amplitude during the first 15 seconds, followed by a slight depression which is maximal at 45 seconds. There is then a rise in amplitude to about 130 per cent of the average test response.

This pattern of response is similar to that obtained by asphyxia with nitrogen (Fig. 2 B). The possibility that the response in Fig. 2 A might be due to asphyxial effects resulting from interference with the respiratory excursions by tubocurarine was excluded in other experiments where the decerebrate preparation was given artificial respiration before the administration of tubocurarine. In such experiments similar effects were found.

Response in decerebrate preparations

If a larger initial dose of curare is given to a decerebrate cat the response is more striking. Fig. 3 shows the effect on the amplitude of the monosynaptic reflex response of the intravenous injection of 1.12 mg. *d*-tubocurarine per kg. body weight. The same initial changes occur, and there is a more marked and longer-lasting increase in amplitude to nearly 400 per cent of the test response.

A further effect of tubocurarine can be observed if a slower sweep is employed. Fig. 4 C shows an irregular repetitive discharge in the ventral roots, which may indicate increased background activity, occurring 1–3 minutes after the intravenous injection of 1.5 mg. *d*-tubocurarine per kg. into a decerebrate cat. The increase of the monosynaptic reflex response does not appear to be related to the appearance

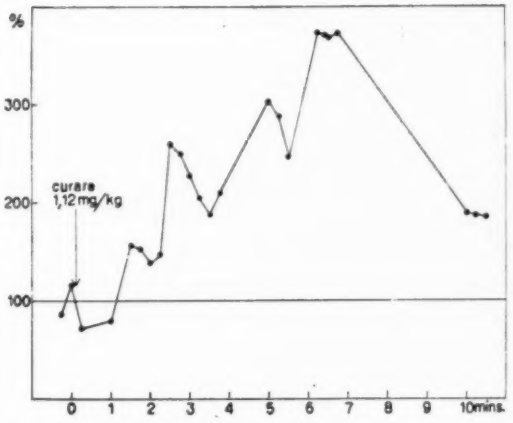

FIG. 3.—Effect of 1.12 mg. tubocurarine per kg. on the amplitude of the monosynaptic reflex response in a decerebrate cat. Ordinates: amplitude as percentage of pre-injection level. Abscissae: time in min.

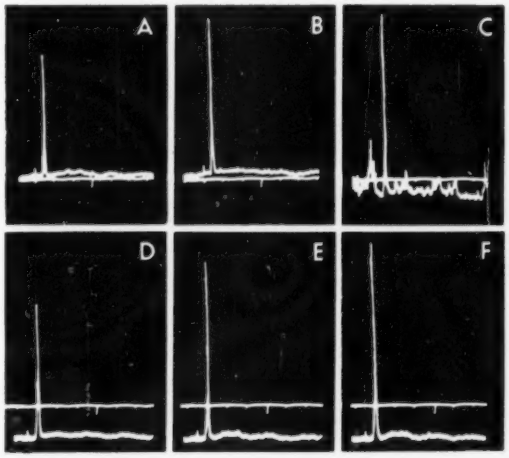

FIG. 4.—The monosynaptic reflex response in S_1 ventral root (A) before, (B) 30 sec. and (C) 1½ min. after, 1.5 mg. tubocurarine per kg. In series D–F, record D was obtained before, E 1 min. and F 2 min. after, a second dose of tubocurarine (1.5 mg. per kg.). Time in 10 msec.

of this activity. In Fig. 4, E and F show the effect of a further injection of the same dose of tubocurarine into the same preparation. There is a similar augmentation of the monosynaptic reflex response, but no sign of repetitive activity. The repetitive discharge in the ventral roots is only seen in decerebrate preparations, usually only after the first and occasionally after the second injection.

Response in spinal preparations

Fig. 5 shows the effect of tubocurarine injected intravenously into a decapitate preparation ; 1.57 mg. per kg. results in an increase in the amplitude of the monosynaptic reflex response to over 200 per cent. A second similar dose 15 minutes later gives a further slight increase in the amplitude. The initial depression observed in decerebrate preparations was seen more seldom in the spinal preparations. Fig. 6 shows a series of records from such an experiment, in which a D.C. amplifier was

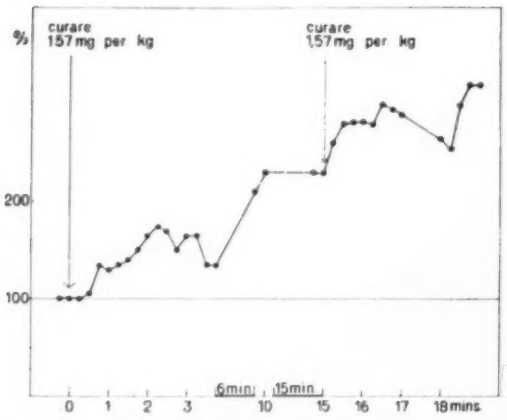

FIG. 5.—Effect of tubocurarine on the amplitude of the monosynaptic response in a decapitate cat in response to 1.57 mg. per kg. at zero time and the same dose 15 min. later.

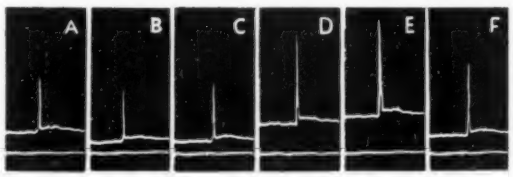

FIG. 6.—The monosynaptic response in L_7 ventral root (A) before, and (B) 25 sec., (C) 30 sec., (D) 1 min., (E) 2 min. and (F) 3 min. after, the injection of 1.7 mg. tubocurarine per kg. Decapitate preparation. Time in 10 msec.

used ; it will be noted that the base line moves up and down as the amplitude of the response rises and falls. This movement represents a change in the membrane potential, which decreases as the amplitude of the reflex response increases. This phenomenon will not be further dealt with in this article.

Fig. 7 shows the effect of smaller doses of curare on a decapitate preparation in which the spinal cord was cut across at the level $T_{11} - T_{12}$. There is again an increase in the amplitude of the reflex response but no early depression.

Fig. 8 shows records from an experiment in which a decapitate cat was subjected to cord section at the level $L_1 - L_2$ and the dorsal roots were all cut from $L_3 - S_3$; the L_6, L_7, and S_1 ventral roots were also cut on one side. The monosynaptic test response was elicited by weak stimulation of the S_1 dorsal root and recorded from the S_1 ventral root. Fig. 9 shows graphically the increase in the amplitude of the monosynaptic reflex response.

In none of the spinal preparations was any spontaneous repetitive activity observed in the ventral roots.

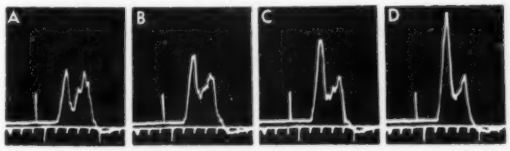

FIG. 8.—The reflex discharge in the S_1 ventral root after stimulation of the S_1 dorsal root (A) before, and (B) 7 min., (C) 11 min., and (D) 18 min. after, the injection of 1.04 mg. tubocurarine per kg. Low spinal preparation. Dorsal roots L_5–S_1 cut.

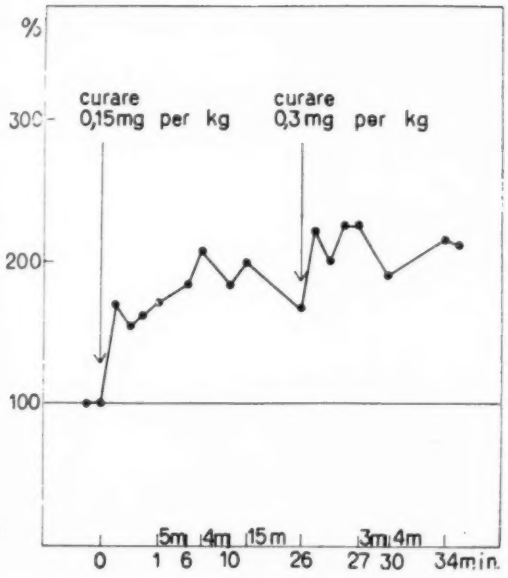

FIG. 7.—Effect of tubocurarine on the amplitude of monosynaptic response in a low spinal cat; 0.15 mg./kg. at zero time and 0.3 mg./kg. 26 min. later.

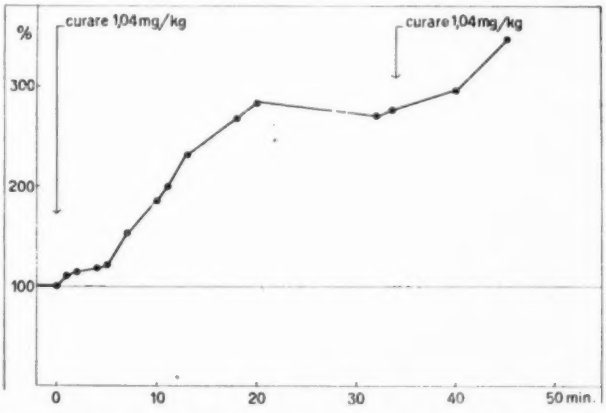

FIG. 9.—Effect of tubocurarine on the amplitude of the monosynaptic response in the L_7 ventral root after stimulation of the L_7 dorsal root in a deafferented low spinal preparation; 1.04 mg./kg. at zero time and the same dose 35 min. later.

Effect on threshold for reflex response

Fig. 10 shows three strength-amplitude curves for the monosynaptic reflex response obtained by stimulation of the nerve to one head of the gastrocnemius muscle in a decerebrate cat. The ordinates represent the amplitude of the reflex response in millivolts, and the abscissae are potentiometric units of stimulus strength. The right-hand curve shows the response to increasing strength of stimulus before the administration of tubocurarine. The middle curve was obtained after the intravenous injection of 0.75 mg. tubocurarine per kg., and the left-hand curve after a total of 3.7 mg. per kg. had been given. It will be observed that the threshold for reflex response remains unchanged, but the curves are increasingly steep, so that a smaller stimulus produces a larger reflex response, once threshold has been reached, after tubocurarine has been administered.

Fig. 11 is a similar curve obtained from a decerebrate cat by stimulating the nerve to one head of the gastrocnemius muscle and recording from the centrally cut S_1 dorsal root. The ordinates represent the amplitude of the earliest afferent volley in millivolts. The abscissae are potentiometric units of stimulus strength. This Figure shows that tubocurarine (0.6 and 1.2 mg./kg.) had no effect on the threshold amplitude of the afferent volleys.

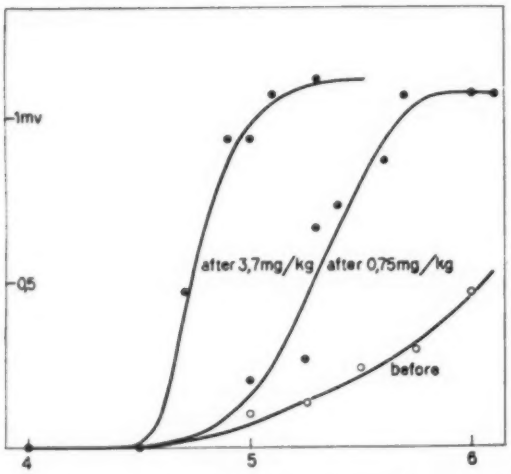

FIG. 10.—Amplitude of the monosynaptic response in mV. plotted against strength of stimulus (nerve to gastrocnemius) in potentiometric units before and after the injection of 0.75 mg. per kg. and 3.7 mg. per kg. of tubocurarine.

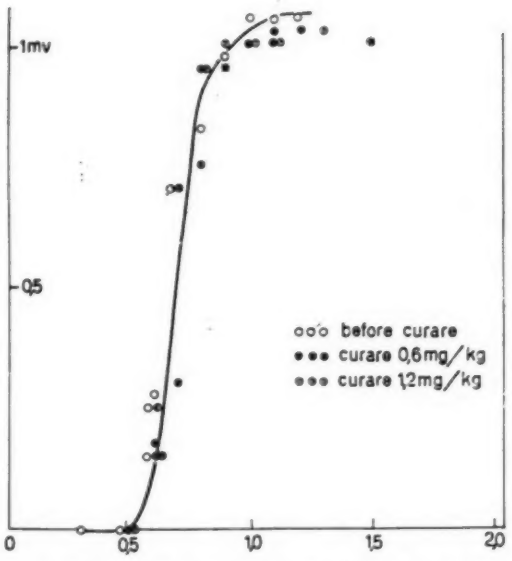

FIG. 11.—Amplitude of the S_1 dorsal root discharge in mV. plotted against strength of stimulus (nerve to gastrocnemius) before and after the injection of 0.6 mg. per kg. and 1.2 mg. per kg. of tubocurarine.

Effect of dial

If dial is injected intravenously 30 minutes before tubocurarine is given the increase in amplitude which is usually seen is either diminished or abolished entirely. Fig. 12 shows the result of such an experiment on a decapitate cat ; 35 mg. per kg. of dial were injected intravenously 30 minutes before the tubocurarine, which was also injected intravenously in a dose of 1.72 mg. per kg. A dose of this order given to a spinal preparation usually gives an increase in amplitude of the monosynaptic response amounting to 150–200 per cent of the average pre-injection test response within five minutes. In this experiment the large dose of tubocurarine given had no effect on the monosynaptic reflex after dial had been given. In other experiments a similar result was obtained, although on some occasions there was some increase in amplitude which, however, was usually short-lived.

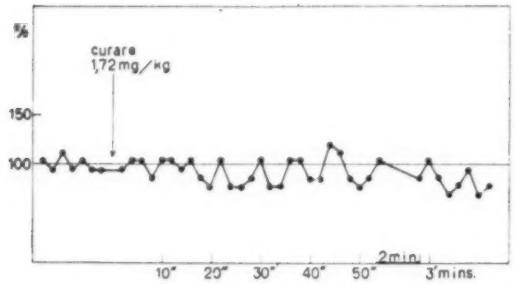

FIG. 12.—Effect of tubocurarine (1.72 mg./kg.) on the amplitude of the monosynaptic response in a high spinal preparation under the influence of dial (35 mg./kg.).

Simultaneous recording of reflex response and blood pressure

It is known that tubocurarine has an influence on the blood pressure, giving a rise after intraventricular or intracisternal injection (see, e.g., Salama and Wright, 1950) and a fall of pressure after intravenous application (see, e.g., Baisset *et al.*, 1949). Since aortic occlusion like asphyxia is followed by a temporary fall and a following increase of the monosynaptic reflexes (see Kirstein, 1951), it was of interest to see if the augmentation of the monosynaptic reflex after intravenous injection of tubocurarine is due to a change in blood pressure. Experiments were therefore performed in which the blood pressure was also recorded. The diagram in Fig. 13

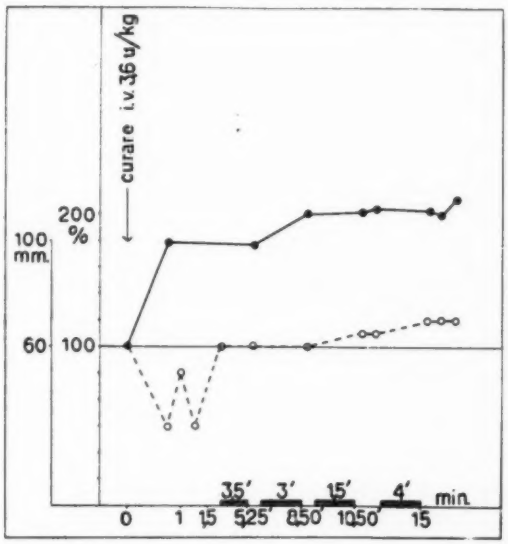

FIG. 13.—Effect of tubocurarine (0.54 mg./kg.) on the amplitude of the monosynaptic reflex response (unbroken line) and the blood pressure (broken line) in a decapitate cat.

shows the results from such an experiment on a decerebrate preparation, the arterial blood pressure being recorded from the common carotid artery. The injection of 0.54 mg. per kg. was followed by a transient fall of the blood pressure during the first two minutes from 60 to about 40, followed by a slow rise to about 70 mm. Hg. The monosynaptic reflex increased to about 200 per cent, and obviously this augmentation developed quite independently of the variations in the blood pressure. When the blood pressure reached its lowest value, 0.5 minute after the injection, the reflex was increased, but the same increase was found also when the pressure had reached the pre-injection value after five minutes.

During the next 10 minutes the blood pressure remained at the pre-injection value and even rose slowly to about 70 mm. Hg. In spite of that the reflex was still augmented to about 200 per cent 15 minutes after the injection. Thus the reflex changes are not related in time to the changes in blood pressure. If, however, in bad preparations the blood pressure was low (about 40 mm. or less) the reflex response became weaker or disappeared. In such preparations the injection of tubocurarine may give a serious drop of the blood pressure followed by a disappearance of the monosynaptic reflex. Such experiments were discarded.

Discussion

It is known that intravenously injected *d*-tubocurarine causes a fall in blood pressure which is partly related to the rate of injection. Baisset, Laporte, and Grezes-Rueff (1949) showed that, when the injection of tubocurarine evokes a sudden and deep fall of the blood pressure, the monosynaptic reflex in the ventral root occurring after dorsal root stimulation may show a transient rise followed by a fall and disappearance of the reflex. This effect, which can be compared with the effect of asphyxia and aortic occlusion (see Kirstein, 1951), was also found in some of our experiments, especially in preparations in which a low blood pressure was recorded before the injections. We found, however, that there is a rise in the amplitude of the monosynaptic extensor reflex which is independent of the transient fall of the blood pressure. Further, the increase of the monosynaptic extensor reflex was shown to persist for a long period after the injection. Our observations are in agreement with the findings of Salama and Wright (1950), who employed the classical technique of reflex study and found that *locally* applied tubocurarine is followed by an increase in the amplitude of the knee jerk. In the decerebrate preparation larger doses of tubocurarine produce spontanous repetitive activity in the ventral roots which appears to be unrelated to the degree of enhancement of the reflex response. It is possible that this effect is related to an influence of tubocurarine on the supraspinal centres as suggested by Salama and Wright (1950).

The experiments on preparations with spinal section at various levels, and on deafferented preparations, indicate that tubocurarine exerts its influence on all levels of the central nervous system and that the augmentation of the reflex response is not attributable only to stimulation of special "facilitatory centres" in the brain.

The fact that Baisset *et al.* did not find any change in the monosynaptic reflex after the injection of tubocurarine which was not correlated to the change of blood pressure may be due to the fact that the blood pressure was about 40 mm. Hg before the injection was given. In the cat at this blood pressure level the drug also has little effect on the reflex response. The negative results of Naess (1950) are apparently due to the use of dial preparations, because we found that dial itself abolishes the response to tubocurarine injections.

The action of tubocurarine on ganglionic and neuromuscular transmission is always depressant, never excitatory. It would be satisfactory to attempt to explain the central effect by a similar blocking action. Alternatively tubocurarine may have central effects totally different from its peripheral action. The phenomenon observed would then be due to a stimulatory effect of the drug on central nervous structures at all levels. This view is difficult to reconcile with the strength-amplitude curve in Fig. 11. This shows that the threshold is unaffected by tubocurarine, and suggests that the increased response is due to an increase in excitability of cells in the subliminal fringe and the recruitment of cells which were previously inexcitable. This might well indicate the removal of inhibitory influences, but is hardly compatible with the idea of a direct excitatory effect of tubocurarine on central structures. In such a case the threshold for reflex response would be expected to fall by a detectable amount.

If the effect on the spinal cord is attributed to a blocking or paralysing action then tubocurarine must have a selective effect on certain structures which normally have a continuous inhibitory action on the monosynaptic extensor reflex. This

concept receives some support from the action of dial, which diminishes or abolishes the central effect of tubocurarine. It has been suggested by Renshaw (1940) that dial depresses interneurone activity, and presumably such depression would make the " inhibitory structures " insusceptible to tubocurarine.

Finally, since both the fall in blood pressure and a liberation of histamine will evoke a secretion of adrenaline it may be argued that the augmentation of the monosynaptic extensor reflex is due to liberated adrenaline. It has earlier been shown that adrenaline influences spinal reflexes (see, e.g., Bülbring and Burn, 1941 ; Bernhard, Skoglund, and Therman, 1947 ; Bülbring, Burn, and Skoglund, 1948). In this connexion it must be emphasized that tubocurarine facilitates the monosynaptic extensor reflex but that we did not find any effect of tubocurarine on the multisynaptic flexor reflex (Bernhard, Taverner, and Widén, 1951) nor on the monosynaptic flexor reflex (unpublished observations). Such an explanation of the action of tubocurarine would imply that adrenaline has a selective effect on the extensor reflex (cf. Bernhard and Therman, 1947 ; Bernhard, Skoglund, and Therman, 1947). The observations that the augmentation of the monosynaptic extensor reflex lasts for a long period (in Fig. 9 more than half an hour) and that the effect is abolished in narcotized preparations (Fig. 13) make it difficult to attribute the effect described merely to the action of liberated adrenaline.

Further experimental results with special reference to the comparison between the central effects of strychnine and curare will be published later.

Summary

1. The results are described of an investigation into the effects of intravenously injected *d*-tubocurarine on the monosynaptic extensor reflex response in the cat in unanaesthetized, decerebrate, decapitate, low spinal, and deafferented preparations and in spinal preparations under the influence of dial. The blood pressure changes were observed and their relationship to the changes in the reflex established.

2. In all the unanaesthetized preparations intravenously injected *d*-tubocurarine was found to increase the amplitude of the monosynaptic extensor reflex. This effect, which was most marked in decerebrate and high spinal and least in deafferented low spinal preparations, was shown not to be correlated with the changes in blood pressure.

3. Spontaneous repetitive activity was observed in the ventral roots of unanaesthetized decerebrate preparations only.

4. Dial injected intravenously before the *d*-tubocurarine was shown to diminish or abolish the effects described.

5. Possible mechanisms of the central action of *d*-tubocurarine are discussed.

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DIFFERENCES IN THE ACTION OF TUBOCURARINE AND STRYCHNINE ON THE SPINAL REFLEX EXCITABILITY OF THE CAT

BY

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In the previous paper dealing with the effect of *d*-tubocurarine on the monosynaptic extensor reflex (Bernhard and Taverner, 1951) the literature on earlier investigations on the central action of curare was reviewed. Among authors who claim that curare has an excitatory central effect Tillie (1890), Santesson (1920–1), von Euler and Wahlund (1941), and Eccles (1946) characterize it as "strychnine-like," whereas McGuigan (1916) was of the opinion that the convulsions produced were not like those caused by strychnine. Cohnberg (1946) believed that curare, in contrast to strychnine, exerted its influence at central nervous levels higher than the spinal cord, and Salama and Wright (1950) came to the conclusion that tubocurarine stimulates the cells of origin of the facilitatory pathways in the brain-stem.

The action of *d*-tubocurarine on the mono- and poly-synaptic reflexes has been studied on anaesthetized cats by McCawley (1949), who found that after large doses both the monosynaptic and polysynaptic reflex responses decreased.

Recently Naess (1950) compared the effect of curare and strychnine on monosynaptic and polysynaptic reflexes and found no effect of curare on either of the two systems, whereas strychnine gave an increase of the polysynaptic response but had no effect on the monosynaptic reflex.

Naess, who worked on dial cats, concluded that "strychnine in smaller doses has no effect, and in greater doses only a depressing one, on the activity of the sensoric and motoric nerve cells which take part in the monosynaptic transmission." Similar results were obtained by Kaada (1950), who tested the effect of strychnine on decapitated cats, and concluded "that after strychnine the two-neurone arc discharge was only slightly increased or even decreased." In contradiction to Kaada and Naess, Bradley and Schlapp (1950) conclude that both "two-neurone and multineurone arc responses are increased by intravenous strychnine" on the basis of experiments on curarized decapitated cats.

Bernhard and Taverner (1951) investigated the effect of varying doses of *d*-tubocurarine on the monosynaptic extensor reflex in decerebrated, decapitated, low spinal, and deafferented preparations as well as on cats anaesthetized with dial.

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They found that *d*-tubocurarine injected intravenously gave an increase in the amplitude of the monosynaptic reflex in the different preparations mentioned. It was also shown that dial abolished the central effect of tubocurarine and that the influence of tubocurarine on the monosynaptic reflex was independent of its action on the blood pressure. Their investigations show the necessity of using different preparations when estimating the central effect of a drug by electrophysiological methods. This will be further emphasized by the results below. In this work the effect of tubocurarine on the polysynaptic reflex has been studied and investigations have been performed on the effect of strychnine on mono- and poly-synaptic reflexes.

METHODS

Decerebrated, decapitated, low spinal, and anaesthetized cats were used. The reflex responses were recorded from the L_7 and S_1 ventral roots, which were divided peripherally and laid on the recording electrodes. The sural and the gastrocnemius nerves were stimulated in order to obtain on the one hand a polysynaptic reflex discharge and on the other hand a monosynaptic reflex discharge in the ventral roots mentioned (Lloyd, 1943). The monosynaptic ventral root responses following stimulation of muscle afferents are impulses returning to the muscle, the afferents of which are stimulated (Lloyd, 1943). Since in the experiments the stimulus was applied to the gastrocnemius nerve a monosynaptic extensor reflex was tested. All exposed nervous structures were covered with warm paraffin oil. Rectangular electrical pulses of 0.1 msec. duration were used as stimuli and were applied to the nerves through electrodes. The ventral root responses were recorded with a differential amplifier and cathode ray oscillograph.

The experimental procedure was as follows. Alternate stimuli of frequency 40–60 per minute were delivered to the gastrocnemius nerve and to the sural nerve. The constancy of the amplitude of the reflex responses was checked for some minutes, and the drug was then injected intravenously and the effect on the amplitude of the test reflexes was recorded. In the diagrams the amplitude of the monosynaptic response is given in per cent of the average pre-injection amplitude. The magnitude of the polysynaptic response was measured planimetrically because of the desynchronization in the polysynaptic reflex discharge, and the planimetrical values are given in per cent of the average pre-injection value. The drugs used were *d*-tubocurarine chloride (Abbott, 3 mg.=20 units/c.c.) and strychnine nitrate (0.9 g./100 c.c.).

RESULTS

Effect of tubocurarine on the polysynaptic reflex

As mentioned above it has been shown that the intravenous injection of *d*-tubocurarine, even in small doses, produces an increase of the monosynaptic extensor reflex response in decerebrate as well as in high and low spinal preparations. Fig. 1 shows the effect of an intravenous injection of a small dose of *d*-tubocurarine (0.18 mg. per kg. body weight) on the monosynaptic extensor reflex response evoked from the gastrocnemius nerve and on the polysynaptic reflex response after stimulation of the sural nerve in a low spinal preparation (decerebrate cat with spinal transection at $T_{12} - L_1$ level). Records A and B show the monosynaptic and polysynaptic responses respectively before the injection of tubocurarine, and records C and D were obtained five minutes after the injection. The monosynaptic response increases, while there is no obvious change in the polysynaptic response. Fig. 2 illustrates the difference between the actions of tubocurarine on the monosynaptic and poly-

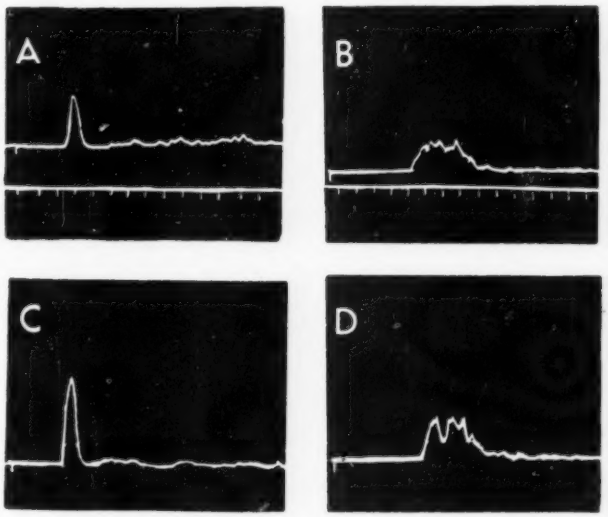

FIG. 1.—Monosynaptic extensor reflex (A and C) and polysynaptic reflex (B and D) in a low spinal preparation before (A and B) and 5 min. after (C and D) the injection of 0.18 mg. tubocurarine per kg. Time in msec.

synaptic reflex responses after repeated injections. The amplitude of the monosynaptic extensor reflex increases after the first injection to about 175 per cent of the pre-injection amplitude, and after the next injection there is a further increase to values higher than 200 per cent (heavy line). There was, however, no significant effect on the polysynaptic reflex (dotted line) even after the last injection. The areas of the polysynaptic responses varied around 100 per cent. When the amplitude of the polysynaptic responses was measured the same result was obtained.

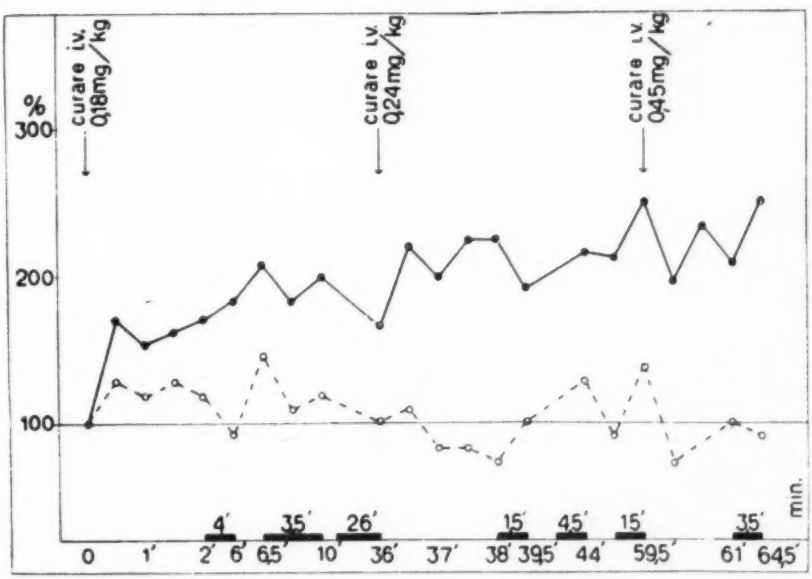

FIG. 2.—Graph showing the effect of tubocurarine on the size of the monosynaptic extensor reflex (heavy line) and the polysynaptic reflex (dotted line) in a low spinal preparation.

A series of experiments was performed on decerebrate and decapitate preparations as well as on deafferented low spinal preparations. In the latter preparation the spinal cord was transected at the level of $T_{12} - L_1$ and the $L_3 - S_3$ ventral and dorsal roots were cut peripherally. The reflex discharge in the L_7 ventral root after stimulation of the L_7 dorsal root was recorded. Varying doses were used from 0.1 to 1.8 mg. per kg. body weight. In all these different experiments tubocurarine gave an increase of the monosynaptic reflex, as described by Bernhard and Taverner (1951), but no effect whatsoever on the polysynaptic response. In anaesthetized preparations (dial) the effect of tubocurarine on the monosynaptic response was abolished (cf. Bernhard and Taverner, 1951). In these preparations the polysynaptic response was the same before and after tubocurarine.

Effect of strychnine on monosynaptic and polysynaptic reflexes

A. *Decapitate preparation.*—Fig. 3 shows the effect of strychnine on the monosynaptic (heavy line) and polysynaptic (dotted line) reflex responses in a decapitate preparation. Before the first point in Fig. 3 three doses of 0.01 mg. strychnine

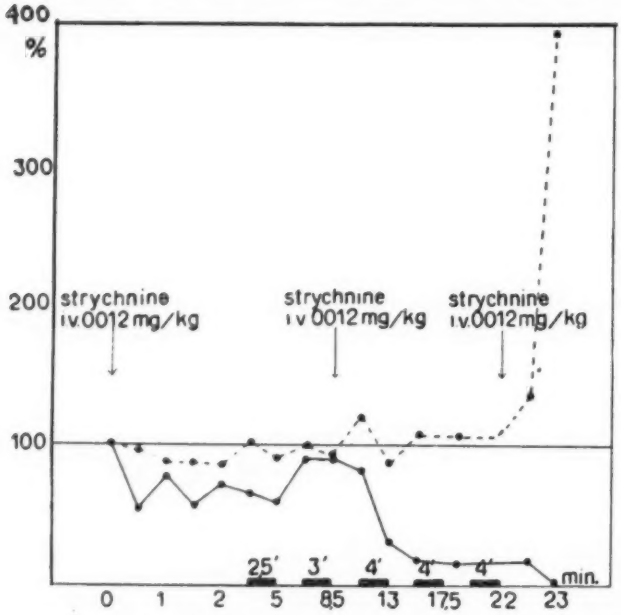

FIG. 3.—Effect of repeated small doses of strychnine on the monosynaptic extensor reflex (heavy line) and on the polysynaptic reflex (dotted line) in a decapitate preparation.

nitrate per kg. body weight were given without any effect on the reflexes. When another 0.012 mg./kg. was injected there was a significant fall in the monosynaptic reflex response, but no change in the polysynaptic reflex response. When the next injection of 0.012 mg./kg. was given the drop in the amplitude of the monosynaptic reflex response was obvious, but not until a further dose of the same size was given was there a sudden increase in the amplitude of the polysynaptic reflex response. After the last injection the experiment was interrupted because the animal developed serious convulsions. The effect obtained on spinal preparations is thus more or less the same as that previously described by Kaada (1950) and Naess (1950), although the decrease in the amplitude of the monosynaptic reflex response is more obvious in our experiments.

If, however, tubocurarine was previously injected the effect of strychnine on decapitate preparations was different. Such an experiment is illustrated graphically in Fig. 4, which shows that the polysynaptic reflex as well as the monosynaptic reflex increases after the injection of 0.08 mg. strychnine per kg. In this experiment 2.25 mg. tubocurarine per kg. was given beforehand. This effect is the same as that reported by Bradley and Schlapp (1950). Obviously the discrepancy between this and the earlier observations depends on the different types of preparation used.

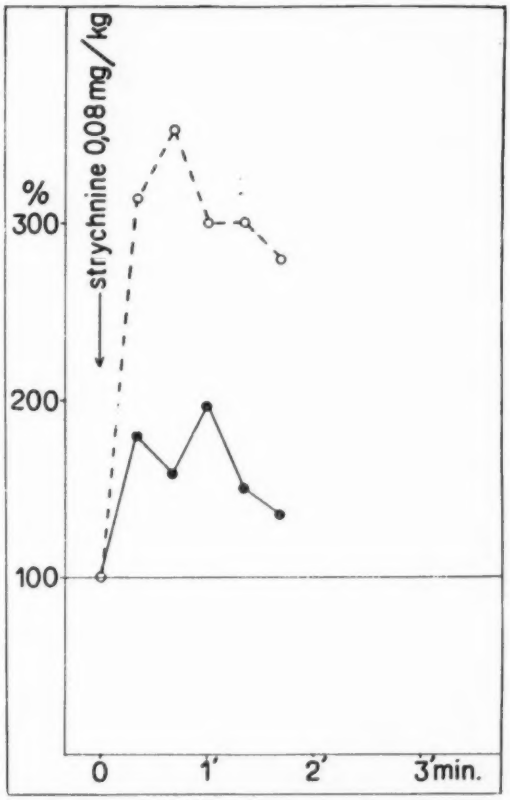

FIG. 4.—Effect of 0.08 mg. strychnine per kg. on the monosynaptic extensor reflex (heavy line) and the polysynaptic reflex (dotted line) in a decapitate cat which had previously received 2.25 mg. tubocurarine per kg.

B. *Low spinal preparation.*—If preparations were used in which the spinal cord was transected at the level of L_1, the effect of strychnine was also different from that obtained on decapitate uncurarized cats. Fig. 5 is taken from such an experiment. In order to avoid convulsions tubocurarine was given previously in a dose of 1.1 mg. per kg. body weight. After the injection of 0.07 mg. strychnine nitrate per kg., both the monosynaptic and polysynaptic reflex responses increased in parallel up to about 200 per cent. A second injection of 0.07 mg. per kg. caused a further rise of both the monosynaptic and polysynaptic reflex responses to 300–400 per cent. One and a half hours after the last injection both reflexes were still about 300 per cent of the pre-injection level. In all experiments on low spinal preparations the same results were obtained, i.e., a concomitant rise of both reflexes. The threshold dose for a significant effect was found to be about 0.06 mg. strychnine

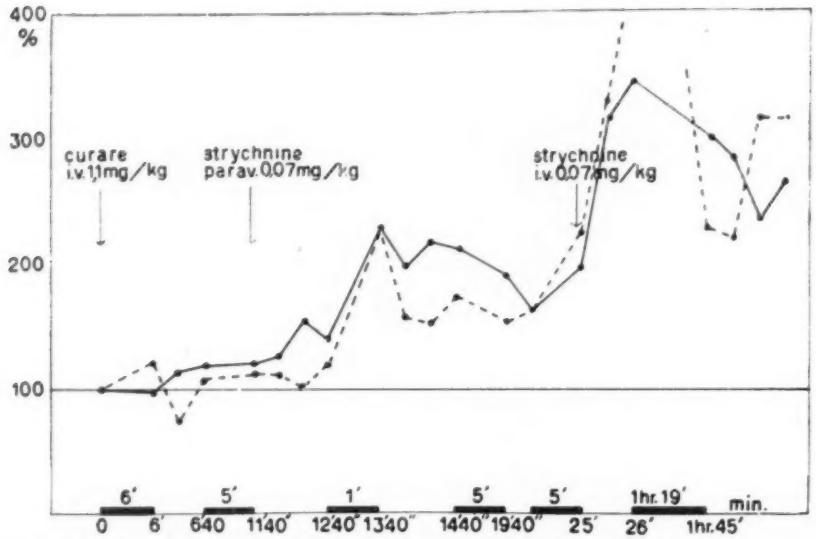

FIG. 5.—Effect of repeated injection of strychnine (0.07 mg./kg.) on the monosynaptic extensor reflex (heavy line) and on the polysynaptic reflex (dotted line) in a low spinal preparation which had previously received 1.1 mg. tubocurarine per kg.

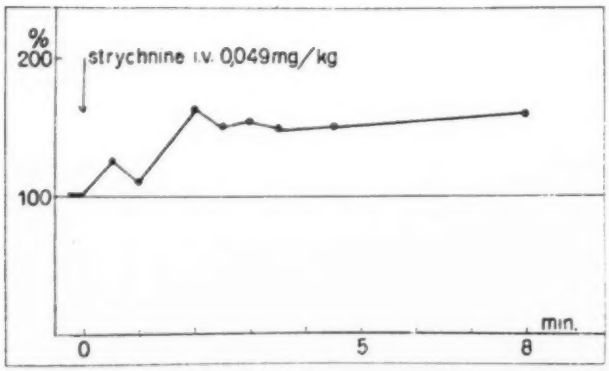

FIG. 6.—Effect of a small dose of strychnine (0.049 mg./kg.) on the monosynaptic extensor reflex in an uncurarized low spinal preparation.

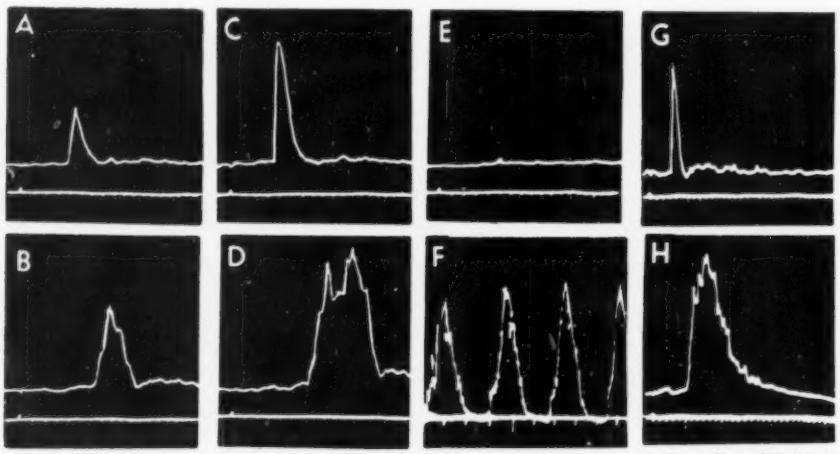

FIG. 7.—Monosynaptic extensor reflex (A, C, E, G) and polysynaptic reflex (B, D, F, H) in a curarized low spinal preparation before (A and B) and after the injection of 0.14 mg. strychnine per kg. In record F the amplification is reduced 50 per cent. Time in msecs. (in record F in 10 msec.). See text for details.

nitrate per kg. The same augmentation of the monosynaptic reflex was obtained in experiments in which tubocurarine was not injected previously (see Fig. 6).

In experiments with doses larger than double the threshold dose synchronized spike outbursts and slow potential waves of varying frequencies occurred ("onde tetaniques provoquées and onde tetaniques spontanées"; Bremer, 1941). In that stage the polysynaptic and monosynaptic reflex responses showed large variations. The records in Fig. 7 from a low spinal preparation illustrate these different effects. Records A and B show the mono- and poly-synaptic reflex responses before the injection. C and D were obtained after the injection of 0.14 mg. strychnine nitrate per kg. and show the increase of the mono- (C) and poly-synaptic (D) reflex responses. Records E and F are from the stage in which the transient spontaneous outbursts occur (steady base line in E, large potential waves in F). As mentioned the reflexes may be abolished in this stage (record E). Records G and H, obtained about one hour after the stage illustrated in E and F, show that the polysynaptic as well as the monosynaptic reflex response is still increased.

Fig. 8 shows the same effect of strychnine on a low spinal and deafferented preparation. The monosynaptic as well as the polysynaptic part of the reflex response from the L_7 ventral root after stimulation of the L_7 dorsal root is increased after the injection of 0.04 mg. strychnine nitrate per kg. (Fig. 8 B).

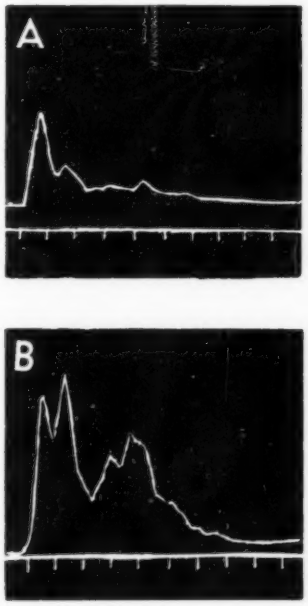

FIG. 8.—Reflex response in L_7 ventral root after stimulation of L_7 dorsal root (A) before and (B) after the injection of 0.04 mg. strychnine per kg. in a low spinal deafferented preparation. Time in msecs.

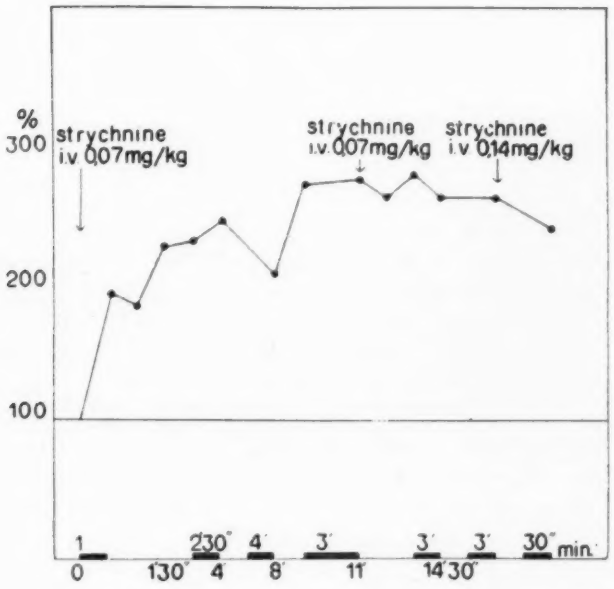

FIG. 9.—Effect of strychnine on the monosynaptic extensor reflex in a low spinal preparation which had previously received 0.45 c.c. dial per kg.

C. *Anaesthetized preparations.*—Fig. 9 shows the effect of strychnine on the monosynaptic extensor reflex response in a low spinal preparation after the previous administration of 0.45 c.c. dial per kg. body weight. There is a rise of the monosynaptic reflex response to more than 200 per cent, which is comparable to the effect in a similar preparation without dial.

Discussion

In an earlier paper (Bernhard and Taverner, 1951) it was shown that intravenously injected tubocurarine produces an increase in the amplitude of the monosynaptic extensor discharge which can be compared with the effect on the knee-jerk of the local application of tubocurarine to the spinal cord, as shown by Salama and Wright (1950). It was also demonstrated that tubocurarine, when injected intravenously, exerts its action at spinal levels as well as on supraspinal structures, and that the steady increase of the reflex is not due to changes in the blood pressure. It was further shown that dial abolishes the effect of tubocurarine.

The experiments described above show that tubocurarine does not influence the polysynaptic discharge in decerebrate, spinal, deafferented, or anaesthetized preparations. Thus, according to our experiments, *d*-tubocurarine acts differently on the monosynaptic extensor and the polysynaptic flexor reflexes.

The action of strychnine on these two reflex systems has also been tested in different preparations. In all types of preparation strychnine gives an increase in the amplitude of the polysynaptic reflex response, thus confirming the results of Kaada and of Naess. In decapitate and decerebrate preparations there is a fall in the amplitude of the monosynaptic reflex response after the injection of strychnine, but in low spinal preparations strychnine even in small doses increases both the monosynaptic and polysynaptic reflexes. This action is not abolished by dial. In decerebrate or high spinal preparations the influence of the increased activity from higher levels brought about by the drug has to be considered. It may be concluded that the augmentation of this continuous activity from higher levels by the action of strychnine is the essential factor in the inhibition of the monosynaptic reflex response in the decapitate preparations (Fig. 3). The findings explain why the enhancing effect of strychnine on the monosynaptic reflex was not noted in earlier investigations (Kaada, 1949; Naess, 1950) in which low spinal preparations were never used. When the cord is transected at the lumbar level the heavy strychnine activity from the upper levels no longer inhibits the monosynaptic reflex response. The change in the reflex discharge is now a sign of the effect of the drug at the segmental level, and obviously the drug augments both types of reflex. Further, it must be pointed out that it still cannot be decided whether the action of the drug is restricted to the neurones included in the reflex arcs or whether it acts on neurone structures in parallel with the afferent neurones which influence the excitability of the motorneurones.

Bernhard and Taverner (1951) pointed out that the augmentation of the monosynaptic extensor reflex after the injection of tubocurarine may depend on the drug blocking structures which normally exert an inhibitory effect on the monosynaptic extensor reflex. It is of interest (i) that strychnine gives an augmentation of the monosynaptic reflex in low spinal preparations in which structures have been

removed which have an inhibitory influence on the reflex, and (ii) that there is also an augmentation of the reflex after strychnine in curarized decapitate preparations (Fig. 4; cf. Bradley and Schlapp, 1950). These observations favour the view that tubocurarine has a blocking action on structures which inhibit the monosynaptic extensor reflex. The augmentation of the monosynaptic extensor reflex after tubocurarine should consequently be regarded as a release.

Summary

1. The effects of *d*-tubocurarine and strychnine on the monosynaptic extensor reflex and the polysynaptic reflex evoked from a cutaneous nerve have been studied in decapitate, low spinal, deafferented, and anaesthetized cats.

2. *d*-Tubocurarine, which is known to produce an increase in the amplitude of the monosynaptic extensor reflex response in unanaesthetized preparations, did not influence the polysynaptic reflex response in any of the preparations tested.

3. Strychnine was found to produce an increase in the amplitude of the polysynaptic reflex response in all types of preparation.

4. It was found that in low spinal preparations strychnine produced an increase in the amplitude of the monosynaptic reflex response parallel to its effect on the polysynaptic reflex, whether or not dial was given previously.

5. When the segment giving rise to the reflex under test is in continuity with the upper part of the spinal cord strychnine produces a decrease in the amplitude of the monosynaptic reflex response; this decrease is interpreted as being due to inhibition of the reflex by an increased continuous activity from upper segmental levels brought about by the strychnine.

6. In the same sort of preparation strychnine gives an augmentation of the monosynaptic extensor reflex if tubocurarine is given beforehand. This fact favours the view that tubocurarine blocks structures which exert an inhibitory action on the monosynaptic extensor reflex.

7. At the segmental level of the reflexes tested tubocurarine and strychnine act differently, i.e., tubocurarine produces an increase in the amplitude of the monosynaptic reflex response but has no effect on the polysynaptic reflex, whereas strychnine increases the amplitude of both reflexes in parallel.

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SOME PHARMACOLOGICAL PROPERTIES OF 3 : 3-DIPHENYL-PROPANOLAMINES, -ALLYLAMINES, AND -PROPYLAMINES

BY

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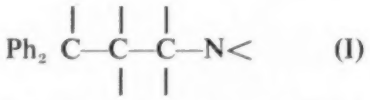
An extensive programme for the synthesis and pharmacological examination of compounds incorporating the structure (I) was initiated in these laboratories in 1946.



(I)

This programme was undertaken with the knowledge that some compounds containing this structure exhibit pharmacological activity. This is exemplified by Höchst 10116 ($Ph_2CH.CH_2CH_2N<[CH_2]_4>CH_2$), which is a component of the anti-asthmatic mixture "Aspasan," in which it increases the effectiveness of dihydroxyephedrine (Schaumann, 1942). There is little clinical literature on its use, but from the amounts manufactured "Aspasan" seems to have been used to a considerable extent in Germany during the war years (B.I.O.S., 1945). The structure (I) is also found in the series of ketones among which amidone ($Ph_2C(CO.Et)CH_2CHMe.NMe_2$) together with closely related analogues is of importance for high analgesic activity, and is now the subject of a very considerable literature. Substituted phenylpropylpiperidines show considerable spasmolytic activity (Becker *et al.*, 1946), and a diphenylpropylpiperidine carboxylic ester ($Ph_2C(CO.OEt)CH_2CH_2N<(CH_2)_4>CH_2$) has spasmolytic and slight analgesic activity (Macdonald *et al.*, 1946).

The present paper is concerned with the pharmacological properties of three series of compounds whose synthesis is described by our colleague Dr. D. W. Adamson (Adamson, 1949). These are the 3 : 3-diphenylpropan-3-olamines (II), 3 : 3-diphenylallylamines (III), and 3 : 3-diphenylpropylamines (IV), where the amino group is primary, secondary, or tertiary, and the quaternary derivatives of the latter.

$Ph_2C(OH)CH_2CH_2NRR'$ (II) $Ph_2C : CH.CH_2NRR'$ (III) $Ph_2CH.CH_2CH_2NRR'$ (IV)

Since our examination of these compounds, Cunningham *et al.* (1949) have found considerable spasmolytic activity in compounds of the type $PhR.C(OH)CH_2CH_2N<(CH_2)_4>CH_2$, particularly where R is phenyl or *cyclo*hexyl ("Artane")

METHODS

The examination of a large number of compounds in several pharmacological tests could only be accomplished with rapid screening methods, and it has therefore frequently been necessary to restrict the use of more precise methods of estimating activity to compounds which have shown high activity in these tests. The following screening methods have been used in compiling Tables I to VI.

Toxicity.—Approximate LD50s by intravenous injection were determined in groups of five mice with a twofold difference between the doses.

Spasmolysis.—Contractures of isolated rabbit ileum suspended in aerated Tyrode-Ringer at 38° C. were induced by carbachol or pilocarpine, the log dilutions or pD values (Miller, Becker, and Tainter, 1948) of these substances being 6.6 and 4.3 respectively. Each test compound at pD 6.0 was added after the spasm was established, and where relaxation occurred lower concentrations were tested. From the relationship between the percentage relaxation and the log dilution an estimate was made of the pD for 50 per cent reduction in spasm, hereafter referred to as the pD50 value. These estimates are subject to very considerable variations between different preparations and are only sufficient in indicate the approximate activity.

Where compounds have shown high activity against carbachol the test was repeated on at least three occasions. The mean estimates are quoted. Some compounds have been tested by direct comparison with atropine on the same pieces of gut, but we would emphasize that unless two compounds have an identical mode of action we cannot expect them to give the same relative activities in all preparations. Tests were also carried out using barium chloride (pD 4.3), but because of the extreme variability of the estimates we have not included these in the Tables.

Antihistamine activity.—This was determined similarly, but on spasm of guinea-pig ileum induced by histamine, pD 6.3. Comparative tests on the same strips of gut were carried out in some instances.

Rabbit eye tests.—The eyes of albino rabbits were used for measuring corneal anaesthetic, mydriatic, and irritant effects. Two rabbits were used for each compound, one eye of each being bathed for one minute with the test solution at 5 mg./c.c. and the other with cocaine at 5 mg./c.c. The reflex to pricking the eye with a fine bristle was tested, and the pupil diameters were measured at five-minute intervals; the illumination was kept constant during the test. The Tables show the mean duration of corneal anaesthesia with the test solutions, and symbols are used to indicate the degrees of mydriasis and irritation. For mydriasis the sign " − " indicates no effect, " ± " a slight but lesser effect than with cocaine, " + " a response equal to that of 5 mg./c.c. cocaine, and " + + " a greater response. For the irritant effect " − " signifies nil, " ± " slight, "+" a definite redness of the conjunctiva, and "++" more severe damage which is frequently associated with increased lacrimation and later perhaps peeling of the cornea. Where estimates of corneal anaesthetic activity have been made we have used the relationship between log dose and log duration described by Young (1951).

Mydriasis by the parenteral route was measured in groups of 10 mice 30 minutes after intraperitoneal injection. At least two doses of atropine sulphate were given on each occasion. Activity in terms of atropine sulphate was estimated by the method described by Ing, Dawes, and Wajda (1945). In general, the fiducial limits for $p=0.95$ are ±10 per cent.

Analgesia.—The effects of each compound on the pain threshold of rats were investigated by the method described by Thorp (1946). No important effects were observed, and no mention of this property is made in the Tables.

A description of other methods used in testing particular compounds is given in the text.

The limits of estimates given in parentheses are the calculated fiducial limits for $p=0.95$.

RESULTS

The pharmacological properties revealed by our screening tests are given in Tables I to VI, where the compounds are classified as follows:

Propanolamines (II)	Amine hydrochlorides, Table I Quaternary ammonium salts, Table II
Allylamines (III)	Amine hydrochlorides, Table III Quaternary ammonium salts, Table IV
Propylamines (IV)	Amine hydrochlorides, Table V Quaternary ammonium salts, Table VI

Propanolamines (II)

Amine hydrochlorides (Table I).—These show fairly powerful atropine-like activity in antagonizing carbachol and pilocarpine spasms of isolated rabbit ileum, and in mydriatic activity by local application in the rabbit and by intraperitoneal injection in mice. While there is no clearly defined correlation between these different aspects of atropine-like function, this does not necessarily imply a true physiological differentiation of function, as different species and modes of application have been used.

TABLE I

PROPANOLAMINE HYDROCHLORIDES

$Ph_2C(OH)CH_2CH_2NRR',HCl$

Serial No.	NRR′	LD50 mg./kg.	Log dilution for 50% antagonism of			Rabbit eye tests with 5 mg./c.c. solutions			Mydriasis (mouse) atropine sulph. = 1
			Carbachol*	Pilocarpine	Histamine	Corneal anaesthesia (min.)	Mydriasis	Irritation	
1	NH_2	90	—	—	—	0	—	—	<0.002
2	NHMe	65	—	—	—	10	—	—	0.0023
3	NHEt	50	—	—	—	0	—	+	0.0044
4	$NHCH_2Ph$	15	—	—	—	35	+	+	
5	NMe_2	100	6.0	—	—	0	—	—	0.0046
6	NMeEt	75	6.5	—	6.1	25	+	—	0.008
7	NEt_2	25	6.7	—	—	20	+	±	0.01
8	NPr^{α}_2	40	—	—	—	10	—	+	0.0022(t)
9	NBu^{α}_2	25	—	—	—	25 (a)	—	++	0.002(t)
10	$NMeCHMeCH_2Ph$	15	6.0	6.0	—	30 (a)	—	±	—
11	$N<[CH_2CH:CH_2]_2$	55	—	·	—	15	—	+	·
12	$N<[CH_2]_3>CH_2$	50	6.9	—	6.2	25	+	—	0.014
13	$N<[CH_2]_4>CH_2$	60	7.6	6.3	6.0	30	+	±	0.02
14	$N<[CH_2]_4>O$	200	—	—	—	·	·	·	—

(t) Mydriasis was accompanied by toxic manifestations. (a) Tested at 2.5 mg./c.c.

* Comparative estimates on the same strips of gut (approximate standard error = ±0.05) except for that of No. 10, which was assumed from the response to one concentration only.

The most active is the piperidino compound No. 13. This, by direct comparison with atropine sulphate on the same strips of rabbit ileum, was found to be about a quarter to a half as active as atropine sulphate in relaxing spasm due to carbachol. By the mydriatic test in mice when the pupil is measured 30 minutes after intraperitoneal injection it is only a fiftieth as active as atropine sulphate.

We have also investigated the effect of this compound on bronchial tone and intestinal movements in dogs, under pentobarbitone sodium, receiving a continuous intravenous infusion of eserine at the rate of 3.2 mg. per hour. The bronchial tone was judged simply from tracings of the amplitude of pressure changes in the tracheal cannula, and movements of the duodenum and ileum recorded with the usual balloon-tambour system. Under these conditions compound No. 13 reduced the bronchoconstriction and inhibited tone and motility of the gut at a dose of 0.1 to 0.2 mg./kg., whereas atropine had a similar action for a longer duration at approximately 0.01 mg./kg. in each of two dogs. Small doses of both compounds commonly produce increased intestinal activity. The spasmolytic activity of this compound has also been examined by Cunningham *et al.* (1949), who found that it showed 36 per cent of the activity of atropine in relaxing spasm of isolated rabbit ileum due to " Furmethide " (furfuryltrimethylammonium iodide). It has a lesser effect by intravenous injection in the dog on furmethide-induced contractions of Thiry-Vella fistulae, on furmethide-induced salivation and on vagal activity, and a smaller mydriatic effect than atropine by local application in the cat.

An investigation of this compound in man (Card, personal communication) has shown that by mouth 25 mg. is ineffective, but 50 mg. reduces gastric motility induced by mecholyl to a lesser extent and for a much shorter duration than a full therapeutic dose of atropine. Card also observed that intravenous injection even of very dilute solutions caused severe thrombosis in man. We were unable to reproduce this by intravenous injection of a 0.5 per cent solution in the rabbit ear, and further when present to the extent of 1 mg./c.c. the compound had no significant effect on the clotting time of horse blood or plasma.

The morpholino analogue (No. 14) is also described by Denton *et al.* (1950), who agree that it is less active than the piperidino compound.

Three compounds of the series, Nos. 6, 12, and 13, showed some antagonism of histamine spasm, but these compounds are very weak antihistamines compared with mepyramine, the pD50 of which is commonly 8.1 to 8.6 under similar conditions.

The antihistamine activity of the propanolamines (II) is less than that of the allylamines (III) and the propylamines (IV). Antagonism of barium spasm was demonstrated with some compounds, and it is likely that such an effect would have been observed with others had higher concentrations been tested, as all members of the group at a log dilution of 4.5 caused inhibition of motility and tone of isolated rabbit ileum.

Some of the compounds (Table I) possess a local anaesthetic activity which, with a 5 mg./c.c. solution, is roughly equal in duration to that of the same concentration of cocaine. Cocaine in our type of test gives a mean duration of 24 minutes with 5.0 mg./c.c. and 70 minutes with 50 mg./c.c., the variation between rabbits being high. Since the local anaesthetic effect of these compounds was frequently accompanied by an irritant action on the conjunctiva, we have not examined this property in more detail.

Quaternary ammonium salts (Table II).—Like other workers, we have found that quaternary ammonium salts are in general more potent than the amine hydrochlorides in atropine-like activity. Several compounds (Nos. 22, 23, 27, 28, and 29) in this series approximate to atropine in activity, the most active being No. 22 (NRR′R″}X = NMeEt$_2$}I), which is described at the end of this section.

TABLE II

PROPANOLAMINE QUATERNARY AMMONIUM SALTS

$Ph_2C(OH)CH_2CH_2NRR'R''\}X$

Serial No.	NRR′R″}X	LD50 mg./kg.	Log dilution for 50% antagonism of			Rabbit eye tests with 5 mg./c.c. solutions			Mydriasis (mouse) atropine sulph. = 1
			Carbachol	Pilocarpine	Histamine	Corneal anaesthesia (min.)	Mydriasis	Irritation	
15	NMe$_3$}I	20	6.5	—	—	10	++	±	0.025
16	NMe$_2$Et}I	20	7.2	6.0	—	0	+	±	0.1
17	NMe$_2$Pr^a}Br	15	7.1	—	—	0	+	±	0.065
18	NMe$_2$Bu^a}Br	15	6.4	—	—	0	++	—	0.023
19	NMe$_2$Ph}I	10	6.6	—	—	5	+	±	.
20	NMe$_2$.CH$_2$Ph}Cl	8	—	—	—	3	—	±	.
21	NMe$_2$.CHMe.CH$_2$Ph}I	5	5.8*	—	—	10	—	—	.
22	NMeEt$_2$}I	20	8.1	7.0	—	0	++	±	0.75
23	NEt$_3$}I	20	7.8	7.2	—	0	++	±	0.45
24	NMePr$^a_2$}I	10	6.2	—	—	0	—	±	0.01
25	NMeBu$^a_2$}I	10	—	—	—	0 (a)	—	—	—
26	NMe[CH$_2$CH : CH$_2$]$_2$}I	10	6.0*	—	—	0	—	—	0.01
27	NMe<[CH$_2$]$_3$>CH$_2$}I	20	8.2	6.6	—	3	+	—	0.35
28	NMe<[CH$_2$]$_4$>CH$_2$}I	10	8.1	6.9	—	25	++	—	0.26
29	NEt<[CH$_2$]$_4$>CH$_2$}I	10	8.3	7.2	—	0	++	—	0.42
30	NMe<[CH$_2$]$_4$>O}I	25	7.5	6.7	—	35	+	+	0.04

(a) Tested at 2.5 mg./c.c.

* Assumed from the response to one concentration.

The other properties of this group are not of particular interest. In general the antagonism of histamine and the local anaesthetic activities are less than in the tertiary amines. Some compounds showed a fairly powerful antagonism of barium-induced spasm of isolated rabbit ileum. No analgesic action was found.

3 : 3-Diphenylpropan-3-oldiethylamine methiodide (Compound No. 22).—In many laboratory tests this compound has shown an activity between 0.5 and 1.0 times that of atropine sulphate. Estimates ranging from 0.7 to 1.0 times atropine sulphate were obtained against spasm induced by carbachol and pilocarpine in comparative tests on the same strip of isolated rabbit ileum. Similarly the mydriatic activity in mice estimated as the unweighted mean for three separate tests was 0.7 times atropine when the pupils were measured 30 minutes after the intraperitoneal injection of the drug. However, the times for onset and duration of action are shorter than with atropine. Fig. 1 shows this in a test on groups of 10 mice when the pupils were measured at several time intervals. The activity of this compound as an antagonist of the depressor effect of carbachol in two cats (under pento-

barbitone), continuously infused with carbachol and adrenaline (Bülbring and Dawes, 1945), was found to be approximately 0.7 times that of atropine. The anti-sialogogue activity was estimated by collecting the saliva from each of six rabbits after the simultaneous injection into opposite flanks of 50 mg./kg. pilocarpine and one of

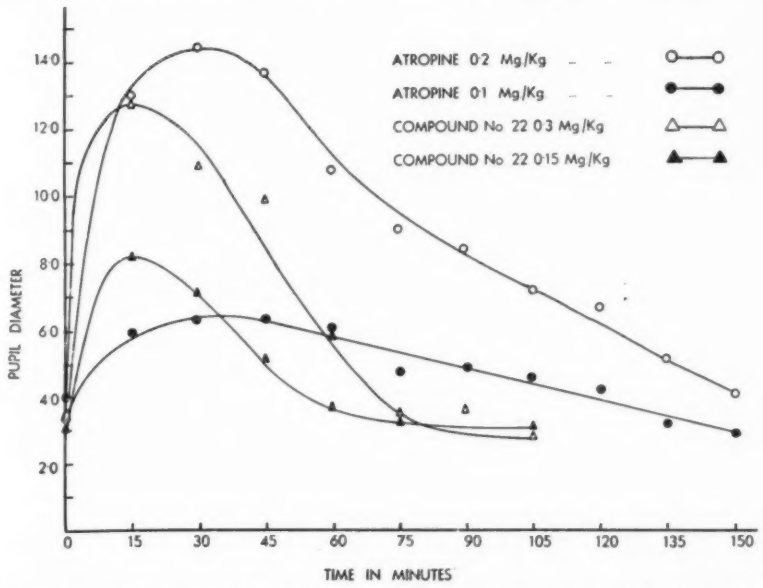


FIG. 1.—The mean pupil diameter (arbitrary units) in groups of ten mice at intervals after intraperitoneal injection of Compound No. 22 and atropine sulphate.

several doses of the antagonist or saline. Compound 22 was found to be 0.6 times as active as atropine (limits 0.23–1.75). Like atropine, and at similar doses, Compound 22 reduces the incidence and severity of the asthma in guinea-pigs produced by exposure to histamine aerosol, and it also inhibits the isolated rabbit ileum and antagonizes barium-induced spasm at pD4 to 4.5. In a dog (under pentobarbitone) 1 mg./kg. intravenously caused a slight transient rise of blood pressure of 20 mm. Hg followed by a transitory fall of 10 mm. Hg. Respiration was unaffected. There was a slight increase in the adrenaline pressor response. In a second dog 0.6 mg./kg. reduced the cardiovascular and salivary effects of 0.6 mg./kg. pilocarpine. The action of doses of between 0.1 and 2.0 mg./kg. on the blood pressure in the pithed dog was irregular. Doses of 0.5 to 2.0 mg./kg. abolished the effects of 0.3 to 1.0 mg./kg. pilocarpine on the blood pressure. The effects of arecoline were also antagonized. The tracing obtained with an intrapleural cannula (Jackson, 1939) showed no evidence of a direct effect on the bronchi in doses up to 2 mg./kg. ; this, however, does not exclude effects when the bronchi are in a state of spasm.

Against spasm of the guinea-pig ileum produced by histamine the pD50 of this compound was 5.2 and that of atropine 5.1 by direct comparison. The intravenous injection of this compound into the guinea-pig 15 minutes before the intravenous injection of histamine reduced the toxicity of the latter. In a test using groups of 10 pigs the LD50 of histamine (1.52 mg./kg. of base in the controls) was increased

1.36 times (limits 0.98 to 1.88) after 5 mg./kg. of No. 22, and 2.6 times (limits 1.7–3.9 after 10 mg./kg. Modification of the effect of histamine on the bronchial system of pithed artificially respired guinea-pigs was shown by an adaptation of the method described by Jackson (1939) for the dog. The initial sensitivity to intravenous histamine was first tested by an injection of 25 or 50 μg. histamine, and only those pigs were used in which a sharp bronchoconstriction occurred. After the histamine antagonist, which was also given intravenously, the dose of histamine required to cause an effect equal to that of the original 25 or 50 μg. was determined. The effect of a given dose of a histamine antagonist was very variable. The dose required for a fivefold decrease in sensitivity in 50 per cent of the test animals was very approximately 0.5 mg./kg. of Compound No. 22 and 0.0025 mg./kg. of mepyramine. Atropine sulphate at 1.0 mg./kg. gave a slight protection, but less than the same dose of Compound No. 22 in another series of tests.

The toxicity in mice of this compound compared with atropine varies with the route of administration.

	LD50 in mg./kg.	
	Intravenous	Intraperitoneal
Compound No. 22	20.5 (19.1–22.0)	114 (104–124)
Atropine	89.0 (83.5–94.5)	245 (220–274)

The difference in toxicity by the two routes is greater for Compound No. 22 and suggests the intervention of some cardiovascular effect on intravenous injection. By mouth, this compound is far less toxic ; 10 mice have survived 300 mg./kg. A dose of 2 mg./kg. has been given intraperitoneally to young rats daily for five days a week for two months with but slight retardation of growth and no macroscopical changes in the internal organs. Intradermally 5 mg./c.c. in the guinea-pig causes a slight transitory reddening at the site of the injection.

No local anaesthetic or analgesic effects have been found.

In man, Compound No. 22 produced atropine effects on the pupil and on salivation. Applied as a 5 mg./c.c. solution to the conjunctiva it caused a dilatation of the pupil within an hour which had disappeared after 12 hours. Its action on the vital capacity in the asthmatic subject was not sufficient for it to be of interest in the treatment of asthma (Herxheimer, personal communication).

Allylamines (III)

Amine hydrochlorides (Table III).—These compounds show less atropine-like activity than the propanolamines (II) but considerable antihistamine and local anaesthetic activity.

The most active antihistamine in this series was found to be the pyrrolidino compound No. 40, which, in a comparative test using four strips from a single guinea-pig, was found to be 0.13 times as active as mepyramine (limits, 0.07–0.23).

Several compounds gave a substantially longer duration of corneal anaesthesia than occurred with an equal concentration of cocaine in the same rabbits. Nos. 32, 37, 38, 40, and 41 showed steeper regression lines relating log dose with log duration than that of cocaine, and they cannot therefore be evaluated in terms of the latter. The following estimates have been made of the concentration for corneal anaesthesia lasting 30 minutes: cocaine 8 mg./c.c., Nos. 37 and 38 approximately

TABLE III

ALLYLAMINE HYDROCHLORIDES

$Ph_2C{:}\,CH.CH_2NRR',HCl$

Serial No.	NRR′	LD50 mg./kg.	Log dilution for 50% antagonism of			Rabbit eye tests with 5 mg./c.c. solutions			Mydriasis (mouse) atropine sulph. = 1
			Carbachol	Pilocarpine	Histamine*	Corneal anaesthesia (min.)	Mydriasis	Irritation	
31	NH_2	45	—	—	—	15	—	++	.
32	NHEt	40	—	—	5.6	55	—	+	.
33	NMe_2	35	6.3	—	6.4	20	—	—	0.0042
34	NMeEt	40	6.5	—	6.9	90	+	+	0.0038
35	NEt_2	30	6.2	5.6	6.4	30	—	±	0.0037
36	NPr^{n}_2	35	—	—	—	30	—	+	.
37	NBu^{n}_2	40	—	—	—	275	—	++	.
38	$NMeCHMeCH_2Ph$	40	—	—	—	>60 (a)	—	+	.
39	$N[CH_2CH{:}\,CH_2]_2$	20	—	—	—	30 (a)	—	++	.
40	$N<[CH_2]_3>CH_2$	100	6.2	—	7.1	55	—	±	0.0039
41	$N<[CH_2]_4>CH_2$	50	6.5	—	6.7	90	±	±	0.0026
42	$N<[CH_2]_4O$	50	—	—	—	15	—	+	.

(a) Tested at 2.5 mg./c.c.

* Comparative estimates on the same strips of gut (approximate standard error = ± 0.05).

TABLE IV

ALLYLAMINE QUATERNARY AMMONIUM IODIDES

$Ph_2C{:}\,CH.CH_2NRR'R''I$

Serial No.	NRR′R″	LD50 mg./kg.	Log dilution for 50% antagonism of			Rabbit eye tests with 5 mg./c.c. solutions			Mydriasis (mouse) atropine sulph. = 1
			Carbachol	Pilocarpine	Histamine	Corneal anaesthesia (min.)	Mydriasis	Irritation	
43	NMe_3	8	6.3	—	—	25	±	—	0.0026
44	$NMeEt_2$	8	7.3	—	6.1	25	+	±	0.015
45	$NMePr^{n}_2$	15	6.5	—	—	25	—	+	.
46	$NMeBu^{n}_2$	35	—	—	—	0	—	+	.
47	$NMe_2CHMeCH_2Ph$	40	6.0*	—	6.0*	20 (a)	—	+	.
48	$NMe[CH_2.CH{:}\,CH_2]_2$	15	6.3	—	—	0	—	±	.
49	$NMe<[CH_2]_3>CH_2$	5	7.2	—	6.1	0	—	—	0.0084
50	$NMe<[CH_2]_4>CH_2$	10	7.5	6.2	6.9	.	.	.	0.018
51	$NMe<[CH_2]_4>0$	10	6.5	—	6.0*	0	—	—	0.003

(a) Tested at 2.5 mg./c.c.

* Assumed from the response to one concentration only.

0.5 mg./c.c., and Nos. 32, 40, and 41 approximately 2.5 mg./c.c. The compounds are much less active than cinchocaine, which has a similar slope to its regression line and gives 30 minutes' anaesthesia at 0.03 mg./c.c. The irritant action and the failure on local injection of 10 mg./c.c. solutions of Nos. 37 and 38 to cause complete

sciatic nerve block in the rat also weigh heavily against any potential value of these compounds as local anaesthetics.

The compounds are not analgesics.

Quaternary ammonium salts (Table IV).—Quaternization again increases the atropine-like activity of some of the tertiary amines. None of the compounds approach the quaternary propanols in activity, but some (Nos. 44, 49, and 50) are a fiftieth to a hundredth of atropine in the mydriatic test and rather more active in antagonizing carbachol *in vitro*. Antihistamine activity in most examples is less than in the amines. It appears from Tables IV and III that the quaternary salt No. 50 is rather more active than the corresponding amine No. 41, but this was not the case in a direct comparison of the two compounds on four strips of ileum from one guinea-pig, when the pD50 of Compound No. 50 was again 6.9 but that of No. 41 was 7.2 and not 6.7. This is an example of the type of variability to which work of this kind is subject.

The compounds are more toxic by intravenous injection in mice than the amines. Local anaesthetic effects are shown to a lesser extent by some members and analgesic effects are again absent.

Propylamines (IV)

Amine hydrochlorides (Table V).—Atropine-like properties such as mydriasis and carbachol antagonism, and antihistamine activity are shown to a degree similar to that in the allylamines (III), but the local anaesthetic activities are less.

The pyrrolidino compound (No. 59) is again the most active of the series against histamine spasm in the guinea-pig. In a comparative test on four strips from one pig which was run in parallel with the test on the allylamine No. 40, Compound No. 59 gave an estimate of 0.12 × mepyramine (limits, 0.07–0.2).

TABLE V

PROPYLAMINE HYDROCHLORIDES

$Ph_2CHCH_2CH_2NRR',HCl$

Serial No.	NRR′	LD50 mg./kg.	Log dilution for 50% antagonism of			Rabbit eye tests with 5 mg./c.c. solutions			Mydriasis (mouse) atropine sulph. = 1
			Carbachol	Pilocarpine	Histamine*	Corneal anaesthesia (min.)	Mydriasis	Irritation	
52	NH_2	90	—	—	—	15	±	+	.
53	NHEt	40	—	—	5.6	5	—	+	.
54	NMe_2	65	6.7	—	6.8	30	+	—	0.0048
55	NMeEt	45	6.2	—	6.8	10	—	±	0.0088
56	NEt_2	25	6.2	—	6.3	5	—	+	0.0045
57	NPr^{α}_2	25	—	—	5.2	25	—	++	.
58	NBu^{α}_2	25	—	—	5.1	45	—	++	.
59	$N<[CH_2]_3>CH_2$	50	6.3	—	7.1	25	±	—	0.0041
60	$N<[CH_2]_4>CH_2$	70	6.8	—	6.7	20	±	+	0.0045
61	$N<[CH_2]_4>O$	80	—	—	—	15	—	±	.

* Comparative estimates on the same strips of gut (approximate standard error = 0.05).

Local anaesthetic action was moderate and frequently associated with irritation. Analgesic activity was not found.

One of these compounds, No. 60 (NRR′=piperidino) also known as "Höchst 10116," was a component of the anti-asthmatic mixture "Aspasan." Its use here was based on the observation (Schaumann, 1942) that a combination of this compound with dihydroxyephedrine gives a more complete protection of guinea-pigs against histamine asthma, and for a longer duration, than either of the two compounds alone; protection against intravenous histamine and antagonism of histamine spasm of isolated guinea-pig ileum were also demonstrated.

Quaternary ammonium salts (Table VI).—This group showed approximately the same level of atropine-like activity (mydriasis and carbachol antagonism) and

TABLE VI

PROPYLAMINE QUATERNARY AMMONIUM IODIDES

$Ph_2CHCH_2CH_2NRR'R''\}I$

Serial No.	NRR′R″	LD50 mg./kg.	Log dilution for 50% antagonism of			Rabbit eye tests with 5 mg./c.c. solutions			Mydriasis (mouse) atropine sulph. = 1
			Carbachol	Pilocarpine	Histamine	Corneal anaesthesia (min.)	Mydriasis	Irritation	
62	NMe_3	12	6.9	—	—	0	+	+	0.02
63	$NMeEt_2$	8	7.3	—	—	25	+	±	0.0044
64	$NMePr^n_2$	10	—	—	—	0	±	—	0.0025
65	$NMeBu^n_2$	10	—	—	—	0	—	—	—
66	$NMe<[CH_2]_3>CH_2$	10	7.5	6.5	—	0	+	—	0.034
67	$NMe<[CH_2]_4>CH_2$	10	7.2	6.6	—	0 (b)	.	—	0.047
68	$NMe<[CH_2]_4>O$	25	6.7	—	—	0	—	—	0.018

(b) Tested at 1 mg./c.c.

toxicity as the quaternary allylamines (Table IV); histamine antagonism and local anaesthetic activity are less in many instances. The most active mydriatics (Nos. 63, 66, and 67) are 0.03 to 0.05 times as active as atropine.

Discussion

The series examined show several associated pharmacological activities which include the atropine-like effects of mydriasis and antagonism of spasm of isolated gut induced by carbachol or pilocarpine, antagonism of the "musculotropic" spasmogen barium chloride, antihistamine, and local anaesthetic activity. The degree to which each of these properties is shown varies between the series. It has been found that atropine-like activity is greatest in the propanols (II), antihistamine activity in the tertiary allylamines (III) and propylamines (IV), and local anaesthetic activity in the tertiary allylamines. The basic group in each series also has an important effect on activity which follows certain general trends in all three series.

Atropine-like activity as manifested by mydriasis and antagonism of carbachol and pilocarpine is frequently greater in the quaternary ammonium salts than in the

tertiary amines. Similar observations have been made in other series, and the significance of this effect has been discussed by Ing, Dawes, and Wajda (1945). These latter workers found that similar quaternary groups favoured high mydriatic activity in benzilic esters as in the three series we have examined. The relative effects of these on activity were slightly different in each series. The tertiary amines show a moderate atropine-like activity which is superior to that of the secondary and primary amines (Tables I, III, and V). Similar tertiary amino groups, namely NMe_2, NMeEt, NEt_2, pyrrolidino, and piperidino, favour activity in the three series, but again to slightly different relative degrees.

Greater differences have frequently been observed between the mydriatic and *in vitro* spasmolytic activities of the tertiary amines than between those of the quaternary ammonium salts. Such differences are frequently due to the test conditions, as with Compound No. 13, which is a fiftieth as active as atropine in the mouse mydriatic test and approximately a quarter to a half as active as atropine as a carbachol antagonist on the isolated rabbit ileum. When tested in the intact dog perfused with eserine, however, its spasmolytic activity was less than a tenth of that of atropine. A similar difference between the spasmolytic activities in isolated rabbit ileum and in the intact dog was observed by Cunningham *et al.* (1949). We wish therefore to emphasize that a true differentiation in function can only be demonstrated when the activities are determined in the same species under similar test conditions.

There is no clear-cut evidence as to the effect of the basic groups on the antagonism of barium-induced spasm in isolated rabbit ileum, but part of this may be due to the inherent variability of the test procedure.

Antihistamine activity is greatest in the tertiary allylamines and propylamines, and in particular where the basic group is NMeEt or pyrrolidino. The pyrrolidino analogues (Nos. 40 and 59) are the most active, but the relative effects of the other groups are not identical in the two series. The activity of secondary amines is not necessarily less than that of the tertiary amines; for example, greater potency occurs when NRR′ is NHEt than when it is NPr_2 or NBu_2. It is interesting that, although the quaternary ammonium salts are considerably less active than the tertiary amines in most examples, the difference is small with some allylamines (e.g., Nos. 41 and 50).

Local anaesthetic activity varies independently of the above effects, and the only general trends with modification in the basic group are a smaller incidence of active compounds among the quaternary ammonium salts than in the tertiary amines and higher activity where the alkyl substituents in the amino group are large. An example of the latter is the high activity where the basic group is NBu_2 (Nos. 9, 37, and 58) or $NMe.CHMe.CH_2Ph$ (Nos. 10 and 38).

The toxicity is also materially altered by modifications in the basic group. A general example is the high intravenous toxicity of the quaternary ammonium salts in mice, compared with the amine hydrochlorides. Different basic groups do not necessarily have the same relative effects on toxicity in the three series.

Pharmacological action varies greatly with the nature of the basic groups in these and other related series, and in any one property in a given species the optimal groups, though commonly allied, may not necessarily be the same even in closely related series. For maximal activity in a given species a compound may need a

defined physico-chemical property, such as a particular dissociation constant, and such a property will be influenced by the basic group, the rest of the molecular structure, and by the medium in which the drug is acting.

Summary

1. The hydrochlorides and quaternary ammonium salts of three closely related series of bases, namely 3 : 3-diphenyl-propanolamines, -allylamines, and -propylamines, have been examined for several pharmacological properties.

2. Atropine-like activity, as manifested by antagonism of carbachol *in vitro* and by mydriasis, is greater in the propanolamines than in the allylamines and propylamines. A very high order of activity is found in the quaternary ammonium salts, and one such compound, namely 3 : 3-diphenylpropan-3-oldiethylamine methiodide, is as active as atropine sulphate on a molecular basis.

3. Antihistamine activity is greater in the allylamines and propylamines than in the propanolamines. The pyrrolidino analogues, which are both a tenth to a fifth as active as mepyramine, are the most potent antihistamines in these series. The quaternary ammonium salts showed various degrees of antihistamine activity, but in general are less active than the tertiary amines.

4. All series showed corneal anaesthetic properties, the degree of activity being greatest in the allylamines ; the most active members are a twentieth as active as cinchocaine and cause considerable irritation of the conjunctiva. The quaternary ammonium salts are in general less active than the tertiary amines.

5. No analgesic activity was found.

6. Minor changes in the basic group frequently have an important effect on pharmacological activity. The relative effects of different basic groups on any one property are not necessarily the same in all series even when the latter are closely related.

The authors are indebted to Dr. D. W. Adamson for his unfailing co-operation throughout this investigation, and also to Miss J. Fawcett, who assisted in the organization of our pharmacological screen. We wish to thank our assistants, Misses E. D. R. Bridge, I. A. Carter, H. Müller, and E. P. Stacey, and Messrs. J. A. Calnan, B. Partridge, and V. C. Sheppard, who carried out most of the pharmacological tests, and Mr. P. A. Young, who analysed some of the results.

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A COMPARISON OF HEAT AND PRESSURE ANALGESIOMETRIC METHODS IN RATS

BY

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WITH AN APPENDIX BY E. I. GODFREY

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In most of the methods which are in common laboratory use for assessing analgesic activities the pain threshold is measured from the response to superficial heat stimulation. The effect of analgesics on pain involving the deeper structures is equally if not more important. Methods for this purpose have been described by Eddy (1928), Haffner (1929), Molitor and Latven (1937), and Friend and Harris (1948). We describe here a simple pressure method, which does not suffer from certain of the disadvantages of these earlier techniques, and compare the results with those obtained by radiant heat stimulation.

METHODS

Pressure method.—The apparatus shown in Fig. 1 was designed to give a uniformly increasing pressure on the tail of the rat. The vertical syringe (A) is connected by transparent "Portex" tubing to the horizontal syringe (B) and to the mercury manometer (C). All air in the system is displaced by a mixture of equal volumes of liquid paraffin and kerosene. At rest, the distance between the head of the plunger of syringe A and the surface of the base of the stand D should be approximately equal to the mean diameter of the tails of the rats. The manometer scale is then adjusted to give zero reading.

With the tail of the rat under syringe A the pressure on it is increased by a uniform depression of syringe B. Readings which are estimates of pain threshold are taken from the manometer when the rat responds—first by struggling, then by squeaking. The pressure is then immediately released.

The nature of the syringe head will have its effect on the observed threshold. In the experiments to be described it was 25 mm. in diameter, slightly concave at the centre, and so rounded that only about 20 mm. of tail was actually in contact with it.

For convenience and standardization we have incorporated a mechanical drive for the horizontal syringe. For the design and construction of this device, which is described in the appendix, we are indebted to Mr. E. I. Godfrey, of these Laboratories. It provides a uniform increase of pressure of 2 cm. mercury per second and an instantaneous release.

Heat method.—The apparatus described by Thorp (1946) for thermal irradiation of the tip of the rat's tail was used; but instead of obtaining thresholds in terms of minimal heat intensity required for a response, we have measured reaction times to a

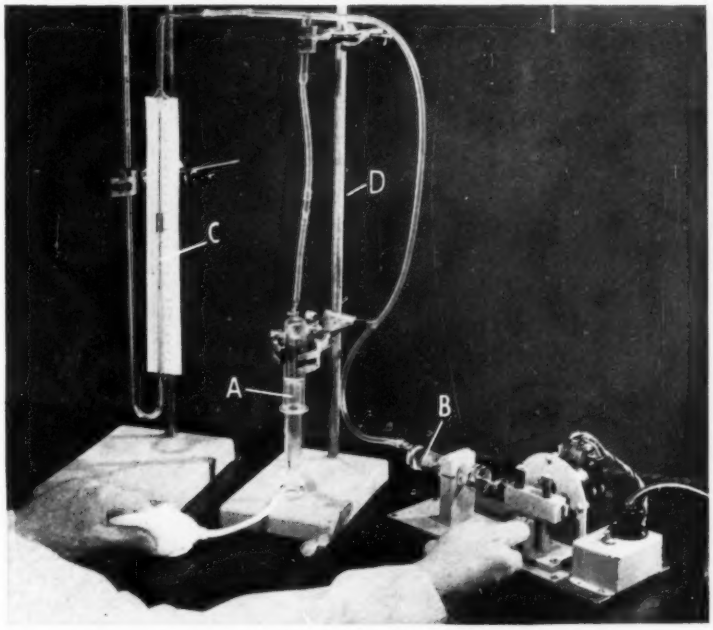

FIG. 1.—The pressure apparatus in use. A and B are syringes connected to the mercury manometer C.

single exposure of constant intensity (100 volts applied to the lamp) as used by D'Amour and Smith (1941).

All experiments were carried out on an inbred strain of Wistar rats.

RESULTS OBTAINED WITH THE PRESSURE METHOD

The normal threshold

The distribution of thresholds.—The normal pressure thresholds in cm. of mercury were examined in groups of 180 rats at several ages. The values were found to be distributed log-normally at each age. Consequently all the quantitative analyses of threshold levels reported below have been carried out on the logarithmic values, although antilogs are quoted for several of the examples for ease of interpretation.

Site of stimulation.—The thresholds determined above were measured at the tip of the tail. It was then found that if pressure be applied at the middle or the base of the tail the thresholds rose in that order. Geometric mean values for struggle and squeak responses of 40 rats of varying age were:

	Struggle	Squeak
Tip	7.3 cm.	8.5 cm.
Middle	10.5 cm.	12.1 cm.
Base	13.2 cm.	14.8 cm.

On practical grounds it was decided to continue observations at the tip of the tail.

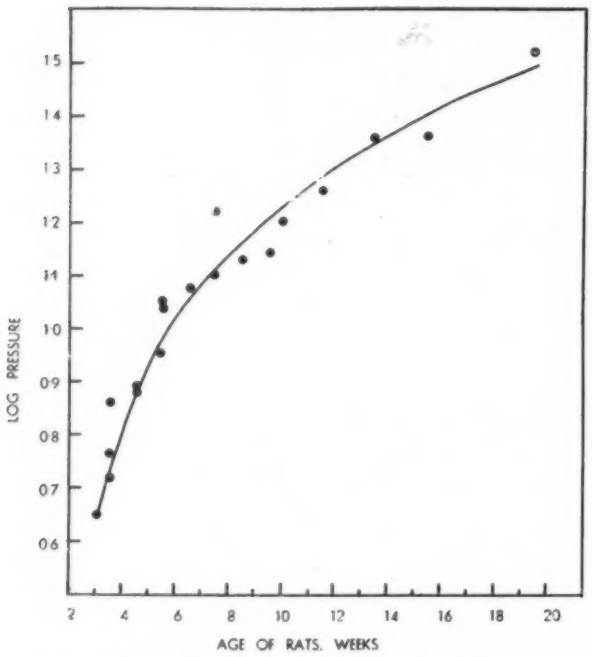

FIG. 2.—The relationship between pressure threshold and age of the rats.

The effect of age.—Three-week-old, newly weaned rats are very sensitive to pressure stimulation, with thresholds of 4–5 cm. mercury. This sensitivity decreases rapidly with age, as reference to Fig. 2 will show ; there, mean thresholds for groups of rats of different ages estimated over several months are plotted as log values. The number of rats per group varied between 20 and 180. Some representative data in this respect are shown in Table I. The ratio of the mean squeak to the mean struggle threshold remains fairly constant at 1.2 for ages of $3\frac{1}{2}$ to $8\frac{1}{2}$ weeks. Thereafter it is subject to rather wider variation, but the overall mean for 3 to $19\frac{1}{2}$ weeks remained at 1.2.

TABLE I

THE PRESSURE THRESHOLDS OF NORMAL RATS AT DIFFERENT AGES

Age, weeks	Number of rats	Mean struggle threshold, cm. Hg	Log. variance (struggle)	Mean squeak threshold, cm. Hg	Log. variance (squeak)	Ratio of means, squeak/struggle
$3\frac{1}{2}$	180	6.53	0.0117	7.87	0.0109	1.20
$4\frac{1}{2}$	144	7.05	0.0051	8.54	0.0084	1.21
$5\frac{1}{2}$	96	9.82	0.0297	12.25	0.0103	1.25
$7\frac{1}{2}$	180	15.39	0.0148	17.78	0.0104	1.16
$8\frac{1}{2}$	100	11.99	0.0204	14.66	0.0133	1.22

The observed values for the rat-to-rat variance differ considerably, but there appears to be a minimum variation between animals of about 4 to 5 weeks old. For general purposes we prefer to use rats between the ages of 3 and 6 weeks with as narrow a range of age as possible in each experiment.

Sex of rats.—No significant difference between the sexes has been observed in rats up to 8 weeks. The males then tend to show rather higher thresholds than females of the same age. It is advisable, therefore, to use equal numbers of each sex per treatment group, particularly with older rats.

Reproducibility of the pain threshold.—Groups of 20 normal rats, at each of three ages, were tested five times at 20-minute intervals. Analysis of variance showed that the variance between trials was never significantly different from the variance between rats, although both tended to be significant against the residual (error) mean square. Similar findings were made when the rats were tested once each morning and afternoon for three consecutive days. The absence of systematic variation among repeated readings showed that with normal threshold pressures no damage was sustained by the tail.

In view of the equal variability between trials and between rats, we might well expect to find no correlation between the individual readings for each rat before and after treatment with an analgesic. Therefore to assess the rise in pain threshold separately for each rat would merely increase the error of the final observations (see below).

The threshold under analgesics

Damage to the tail.—No obvious physical damage to the tail has been observed in rats treated with analgesics after the application of a pressure of four times the control value, our general "cut-off" pressure, but in an experiment using 3-week-old rats injected with 8 mg./kg. morphine sulphate a pressure of eight times the normal threshold caused bruising. (These animals were not allowed to recover.) Stimulation repeated every 15 minutes after treatment with morphine appears both to shorten the apparent duration of the drug action and to depress the threshold upon recovery. Such hypersensitivity may sometimes be detected as long as 24 hours after injection by a decrease in the normal threshold of approximately 15 per cent.

Correlation with the normal threshold.—In a test on 20 3½-week-old rats the mean "squeak" threshold was initially 7.4 cm. and after 2.5 mg./kg. of morphine subcutaneously was 13.8 cm. The coefficient of correlation for the 20 pairs of individual observations was estimated as:

$$r = +0.116, \text{ from which } p = 0.65$$

Hence there was no significant correlation between the initial values and corresponding thresholds after treatment.

Since there is no statistical advantage to be gained from the measurement of individual elevation of threshold, only post-injection readings need be made on each rat, and the design of quantitative tests is simplified.

The standard response curve.—A typical dose-response curve for morphine sulphate injected subcutaneously into 7-week-old rats is shown in Fig. 3. The thresholds were measured 30 minutes after injection, and each point represents the mean of the log thresholds for struggle and squeak of 10 rats. None of the rats injected with 16 mg./kg. responded at or below the cut-off pressure of 50 cm. Hg. The curve shows significant flattening between 1 and 2 mg./kg. Between 2 and 8 mg./kg.

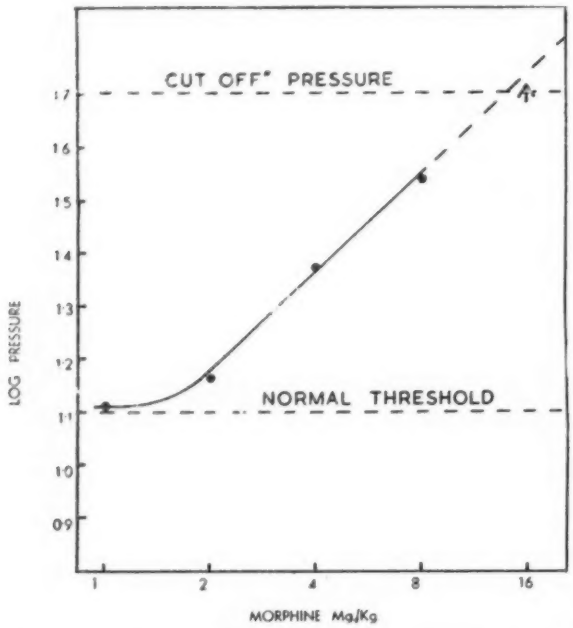

FIG. 3.—The elevation of the pressure threshold by graded doses of morphine sulphate. Mean thresholds for groups of ten rats at 30 minutes after subcutaneous injection.

the regression is linear with a regression coefficient of 0.625, S.E. ± 0.052. The standard deviation of a single observation in this experiment was ± 0.070 (equivalent to approximately ± 16 per cent).

Comparative tests with the pressure method

Quantitative technique.—It is possible to conduct comparative tests of unknown compounds against morphine as a standard. The results of such a test, and their analysis, are presented in Tables II and III. In this experiment the compound

TABLE II

RESULTS OF A COMPARATIVE TEST OF MORPHINE AND 191C49

Values are the means of struggle and squeak thresholds for each rat measured 30 minutes after injection

	Morphine sulphate (mg./kg.)		191C49 (mg./kg.)	
	2.0	3.0	2.0	3.0
Pain thresholds, cm. Hg	10	20	8	11
	9	12	4	12
	12	10	12	16
	6	20	6	8
	9	16	7	13
	12	14	10	6
	14	14	8	18
	9	17	8	15
	11	20	12	19
	16	12	14	14
Logarithmic means, cm. Hg	10.4	15.1	8.4	12.5

TABLE III

ANALYSIS OF VARIANCE OF LOG THRESHOLDS OBSERVED IN THE COMPARATIVE TEST OF MORPHINE AND 191c49

Source of variation	n	Mean square	F	p
Between drugs	1	0.07744	4.04	0.05
Between doses	1	0.27855	14.53	<0.001
Departure from parallelism ..	1	0.00046	—	—
Residual (error)	36	0.01916		

Common slope, b = 0.916, standard error ±0.249

191C49, 3-diethylamino-1 : 1-di(2-thienyl)but-l-ene hydrochloride (Adamson and Green, 1950), was compared with morphine sulphate in 3½-week-old rats. The mean threshold before treatment for the 40 rats was 5.73 cm. Hg ; the values 30 minutes after injection are shown in Table II. While the log-threshold/log-dose lines for the two drugs are shown to have significant regressions and to be parallel, the standard error of the combined slope is rather large. Taking this into account, the ratio of activity is found to be 0.80 × morphine with fiducial limits for p = 0.95 of 0.53 – 1.00 × morphine sulphate.

Greater precision could be attained by increasing the number of rats tested or by increasing the dose interval to reduce the standard error of the slope. The latter alternative is not always practical for the reason given below.

Quantal technique.—As other workers have found in using different methods of stimulation, it has been our experience that the quantitative technique is frequently invalidated by some animals in a group treated with an analgesic failing to respond to pressures of about four times the control value. Any statistical device of substituting positive values for "insensitive" rats such as devised by Ipsen (1949) is unreal, and we prefer to use a quantal interpretation of results. Arbitrarily, an animal is considered to show positive "analgesia" when its threshold is at least twice the mean value of a control group. A standard probit analysis is possible on such results, an example of which is illustrated in Fig. 4 and Table IV. At the foot of the latter are quoted the fiducial limits of the relative potencies of the several compounds in terms of morphine sulphate 30 minutes after injection.

While the ratio quoted for phenadoxone is in agreement with the value found by Basil, Edge, and Somers (1950) in the rat, the amidone value appears somewhat diversely in the literature. Thus Scott, Kohlstaedt, and Chen (1947)—see also Chen (1948)—found that the potency of amidone was about twice that of morphine sulphate in nullifying the pain from pinching the rat's tail, whereas the majority of experimenters who have used radiant heat stimulation have reported that amidone is 1 to 1.3 times morphine (Thorp, Walton, and Ofner, 1947 ; Cahen, Epstein, and Krementz, 1948 ; Thorp, 1949 ; Hougs-Olsen, 1949 ; Bonnycastle and Leonard, 1950). A notable deviation from the heat ratios is the value of 3.4 : 1 found by Tainter and Buchanan (1949). Because at the time it seemed that the relative activity of amidone and morphine might be related to the type of stimulus used, an extensive comparison of these compounds by the heat and pressure methods was begun here in 1948.

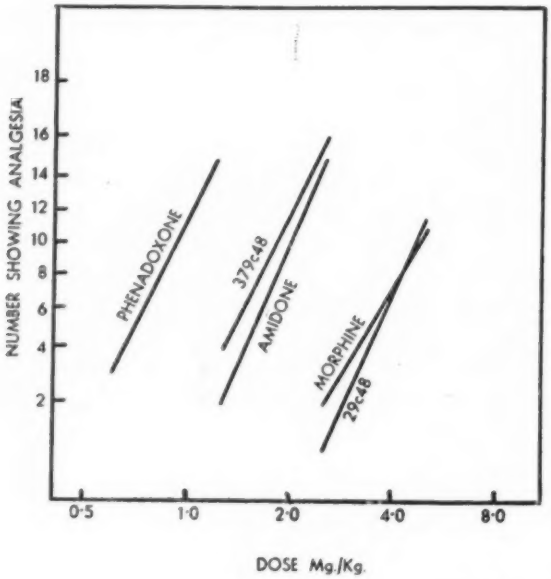

FIG. 4.—Probit response lines of five drugs by the pressure method. Groups of twenty rats were used; details of the analysis of this experiment are given in Table IV.

TABLE IV

SOME ITEMS IN THE QUANTAL ANALYSIS OF A COMPARATIVE TEST BY THE PRESSURE METHOD USING A GROUP OF 20 RATS AT EACH DOSE

Drug	Morphine sulphate	Phenadoxone	29C48*	379C48†	Amidone
Dose, mg./kg.	2.5 5.0	0.6 1.2	2.5 5.0	1.25 2.5	1.25 2.5
Rats showing "analgesia"	2 11	3 15	1 12	4 16	2 15
Weighted mean log dose ..	0.4965	−0.1514	0.5206	0.1505	0.1847
Weighted mean probit response	4.63	4.92	4.74	5.00	4.92
Slope	4.65	5.65	6.31	5.32	6.64
Std. error of slope ..	±1.38	±1.52	±1.82	±1.50	±1.63
Log potency	0	0.699	$\bar{1}$.995	0.411	0.363
Variance of log potency ..	—	0.00321	0.00344	0.00320	0.00338
Ratio to morphine ..	1	5.00	0.99	2.58	2.31
P.95 limits of ratio ..	—	3.87–6.46	0.76–1.29	2.00–3.33	1.77–3.00

χ^2 for homogeneity of slopes = 0.952, 4 d.f., p = 0.91. Common slope = 5.66, std. error ±0.715.

* 6-Piperidino-4: 4-diphenyl-5-methyl-3-hexanone hydrobromide. † 6-Piperidino-4: 4-diphenyl-3-heptanone hydrochloride. (Ofner, Thorp, and Walton, 1949; Ofner, Walton, Green, and White, 1950.)

THE COMPARISON OF PRESSURE AND HEAT RESULTS

The normal thresholds

We have found the reaction times to heat stimulation, like the pressure thresholds, to be log-normally distributed in our experiments. Confirmation in regard to heat reaction times may be found in the data published by Ercoli and Lewis (1945). A similar frequency table given by D'Amour and Smith (1941) does not conform to either the normal or log-normal type, but this may be due to the mixed population of rats which these workers used. The logs of the heat reaction times have been used in the analyses described in this paper.

Experiments on normal rats showed that when equal areas of the tail were irradiated the sensitivity to heat was greatest at the tip. In contrast to the pressure thresholds, heat reaction times showed no definite change with age, although they frequently tended to be shorter in the older rats.

A comparison of the variances by the two methods showed that the heat reaction times were more variable on different occasions in groups of the same age than were the pressure thesholds, but that the internal variances of each were of the same order. Further, the variance of the heat reaction times like the variance of pressure thresholds was approximately the same between observations made at 20-minute intervals as between rats.

In order to justify our later comparative tests with analgesics, an experiment was conducted on 40 rats in which both pressure thresholds and heat reaction times were observed on each rat with the minimum delay between the two measurements (approximately 15 seconds). Alternate animals were subjected to pressure first and heat first. Analysis of variance showed that neither measurement was affected by the order in which they were determined.

The relative activity of morphine and amidone

All the tests in this section have been carried out in the following manner. Four doses of each drug have been injected subcutaneously into groups of 10 or 20 rats, while a control group received injections of saline. A twofold dose interval was used throughout. Pain thresholds to heat and then to pressure were determined before and 30 minutes after treatment. The mean threshold for the saline group was never significantly different before and after treatment ; the latter estimate was used as the control value in assessing quantal responses. Probit analyses showed that within every test the regressions for the two drugs were parallel, although the slopes varied from test to test. It was possible to compute for each experiment

(*a*) the ED50, being the dose required to produce " analgesia " in 50 per cent of the animals, and

(*b*) the ratio of the ED50 value for amidone compared with that for morphine.

Tests were performed on two series of rats. The first series were tested at $3\frac{1}{2}$ weeks of age, and then fortnightly until $19\frac{1}{2}$ weeks old, the rats being randomized between successive occasions. The second series consisted of groups of previously unused animals at various ages from $4\frac{1}{2}$ to 14 weeks.

It should be mentioned that at ages above two months rats become difficult to handle and their responses poorly defined, particularly by the pressure method. This has been reflected to some extent by the variation of the results. The estimates of ED50 shown in Table V varied for both drugs and in both series. In the series of previously unused rats the ED50 increased between the ages of $3\frac{1}{2}$ and 10 weeks, which has been our general experience in many other tests. A similar trend was seen in the rats which were repeatedly injected, but even after the first treatment the sensitivity to the analgesics seems to have decreased, suggesting the development of some degree of tolerance. A similar comment was made by Ercoli and Lewis (1945). The divergence between the two series did not, however, increase with the infrequent injection given. The overall mean estimate for the ED50 in the previously used rats is twice that in the fresh animals, as shown in Table VI.

TABLE V

THE ED50s (MG./KG.) FOR MORPHINE AND AMIDONE IN RATS OF DIFFERENT AGES ESTIMATED 30 MINUTES AFTER SUBCUTANEOUS INJECTION

Age in weeks		Amidone			Morphine sulphate		
Series I	Series II	Pressure-struggle	Pressure-squeak	Heat	Pressure-struggle	Pressure-squeak	Heat
3½		1.3	1.3	1.1	2.9	2.7	3.3
	4½	1.6	1.5	1.0	3.6	3.8	2.1
5½		2.0	3.2	2.0	5.1	5.6	4.6
7½		3.1	2.8	2.4	7.2	7.4	4.7
	8½	1.7	1.7	1.5	3.5	3.6	3.6
9½		2.7	3.7	2.5	8.1	6.9	7.1
	10	2.0	1.9	1.1	5.5	4.3	2.7
11½		2.6	2.6	3.0	6.7	5.7	8.2
13½		2.1	2.2	4.8	4.9	4.8	4.8
	14	1.0	1.0	1.6	1.8	2.0	2.3
	14	2.0	2.2	3.3	3.1	2.8	7.6
15½		7.0	4.1	6.0	24.0	10.6	10.2
19½*		2.3	2.4	2.8	8.2	8.8	7.1

* Tests on Series I were not carried out at 17½ weeks of age.

TABLE VI

UNWEIGHTED LOG MEAN ED50s FOR MORPHINE AND AMIDONE. "UNUSED" RATS REFER TO SERIES II OF TABLE V AND THE 3½-WEEK OBSERVATIONS ON SERIES I. "USED" RATS REFER TO THE REMAINDER OF THE FIRST SERIES

Rats	Amidone			Morphine sulphate		
	Pressure-struggle	Pressure-squeak	Heat	Pressure-struggle	Pressure-squeak	Heat
Unused	1.6	1.6	1.5	3.2	3.1	3.2
Used	2.8	2.9	3.1	7.9	6.9	7.5

There it will also be seen that the ED50s for struggle and squeak responses are identical. It follows that the correlation observed between these two thresholds in normal rats is maintained after treatment with these analgesics. For this reason only the squeak estimates have been used in calculating activities. While the mean ED50 for all groups was the same for pressure and for heat responses, the values differed significantly between tests. The ratios of activity of amidone and morphine found are listed in Table VII.

It was possible to show statistically that these ratios were completely homogeneous throughout all the tests. The age of the rats, their previous usage, and the method of stimulation produced no significant variation of the values. In particular the mean log ratios for pressure (squeak) and heat were:

Pressure 0.3396, variance 0.0004037
Heat 0.3385, variance 0.0004796

From these we obtain for the difference of the two estimates,

$$t=0.64,\ p=0.65$$

TABLE VII

RATIOS OF ACTIVITY OF AMIDONE AND MORPHINE SULPHATE, MEASURED BY PRESSURE AND HEAT STIMULATION. THE WEIGHTS ARE THE RECIPROCALS OF THE VARIANCES OF THE LOG RATIOS

Age in weeks		Pressure				Heat			
Series I	Series II	Ratio		Weight		Ratio		Weight	
3½		2.1		221		3.0		144	
	4½		2.5		340		2.0		139
5½		1.8		185		2.3		104	
7½		2.7		260		2.0		537	
	8½		2.1		289		2.2		173
9½		1.9		224		2.8		240	
	10		2.3		213		2.3		118
11½		2.2		215		2.7		146	
13½		2.2		219		2.5		167	
	14		2.0		55		1.4		96
	14		1.3		117		2.3		43
15½		2.6		103		2.0		77	
19½		3.1		60		2.6		101	

There being no significant difference, the grand mean ratio may be quoted as amidone$\equiv$2.23 $\times$ morphine sulphate, with fiducial limits for p=0.95 of 2.08 – 2.39. Before this particular comparison of amidone and morphine, several other comparative tests had given a mean activity ratio of 2.38 with a log variance of 0.0003390. Combining the two values gives an estimate for the ratio of 2.29 with fiducial limits for p=0.95 of 2.17 – 2.41.

The activity of pethidine

When tested by various heat methods the analgesic activity of pethidine in the rat seems to vary under different conditions. For example, the following estimates of the pethidine/morphine ratio have been reported: 0.05 to 0.1 (Bonnycastle and Leonard, 1950), 0.14 (Cahen, Epstein, and Krementz, 1948), 0.15 (Thorp, Walton, and Ofner, 1947), and 0.2 (Tainter and Buchanan, 1949). The last authors named also quote Bliss and Sevringhaus (1947) as having analysed the results of a collaborative study in which several laboratories participated and found that the relative potency of pethidine varied between 0.05 and 0.45 times that of morphine. One possible explanation of this variation is to be found in the observation made by Davies, Raventos, and Walpole (1946) that the slope of the pethidine regression line was only half that of morphine. Because of the variation in the relative activity of pethidine by the heat method, a comparison of the heat and pressure ratios may only be made when the two effects are measured in parallel.

We have compared pethidine hydrochloride and morphine sulphate in a similar manner to that already described for the comparison of amidone and morphine. Six experiments were carried out, and in each about 120 rats were used and two or more doses of each analgesic were injected. The estimated slopes of the regression lines and activity ratios are shown in Tables VIII and IX respectively.

By the heat method the slopes of pethidine and morphine were significantly different in only one of the six tests, while by the pressure method no significant

TABLE VIII

THE SLOPES OF THE REGRESSION OF PETHIDINE HYDROCHLORIDE AND MORPHINE SULPHATE BY THE QUANTAL TECHNIQUE

"p" = probability of differences occurring by chance ("t" test)

Exp. No.	Heat method			Pressure method		
	Slopes		"p"	Slopes		"p"
	Pethidine	Morphine		Pethidine	Morphine	
1	2.79	2.58	0.85	3.36	3.61	0.85
2	1.79	3.85	0.20	5.34	5.00	0.85
3	0.86	4.97	0.015	2.72	4.02	0.40
4	2.99	2.33	0.75	3.65	4.65	0.65
5	2.99	6.31	0.25	6.64	3.99	0.35
6	7.06	13.08	0.15	4.44	7.91	0.075
χ^2 between experiments ..	7.79	10.06	—	5.17	7.62	—
p	0.15	0.075	—	0.40	0.20	—
Weighted means	2.43	3.88	0.10–0.05	3.75	4.94	0.10
Variance of means ..	0.240	0.368	—	0.204	0.321	—

TABLE IX

THE ACTIVITY RATIO OF PETHIDINE HYDROCHLORIDE : MORPHINE SULPHATE BY THE HEAT AND PRESSURE METHODS

"p" = probability of difference of log ratios occurring by chance ("t" test)

Exp. No.	Heat method ratio	Pressure method ratio	"p"
1	0.11	0.15	0.30
2	0.12	0.20	0.05–0.02
4	0.18	0.44	<0.001
5	0.24	0.46	0.01
6	0.25	0.29	0.35
χ^2 between experiments	19.7	41.8	—
p	<0.001	<0.001	—
Weighted geometric means ..	0.20	0.27	0.001

differences were found. The slopes were homogeneous between tests for each drug by each method, and the four means showed that the pethidine slope was more shallow than the morphine slope by both methods. The probability of the observed differences between the means occurring by chance was low, although not significant at the 5 per cent level.

By using the respective weighted mean slopes in the five tests not showing internal significant differences, the pethidine/morphine activity ratios shown in Table IX were calculated. By neither method were these ratios homogeneous, and in each experiment pethidine was more potent by the pressure than by the heat method. The five differences between the (log) ratios were homogeneous ($p=0.10$), and the mean of these indicated that the pressure ratio was about 1.5 times the heat ratio with probability limits of 1.25–1.85 ($p=0.95$).

From the foregoing evidence it would appear unlikely that the pethidine and morphine regressions are in fact parallel, and no single estimate of relative potency would therefore be valid, either for heat or pressure. The mean ratios quoted in Table IX represent only the order of potency under the conditions required to elevate the pain threshold by 100 per cent in 50 per cent of rats.

Discussion

The heat and pressure methods are similar in that the intensity of stimulus may be assumed to increase nearly linearly with time. Whereas in the heat method it is largely the superficial layers of the tail which are stimulated, in the pressure method the deformation of the deeper structures is presumably the chief cause of excitation.

Both thresholds were found to be log-normally distributed, and a study in normal animals showed that, whereas heat reaction times tended to decrease with increasing age, the pressure thresholds very markedly increased. This may be explained largely by physical considerations in so far as the area exposed to heat irradiation increases with the diameter of the tail, whereas the pressure per unit area decreases. The greatest increase in the normal pressure thresholds with age was concurrent with the period of maximal rate of anatomical development of the tail.

A point of some theoretical interest arises from the observation that the normal thresholds for heat reaction time and " struggle " have a greater variance than that for " squeak." This may be correlated with the behaviour pattern of a rat when a painful stimulus is applied to the tail, since the animal endeavours to remove its tail from the source of stimulation, and only when this is unsuccessful does it squeak " in protest." We may draw a parallel with some unpublished experiments carried out in man at these laboratories in collaboration with Dr. A. C. White, where thresholds for five degrees of pain ranging from " discomfort " to " intolerable " were determined by applying pressure to the base of the finger-nail with a modification of the analgesic apparatus described in this paper. The analysis of 90 observations involving 8 subjects showed that the variances decreased as the degree of pain increased. The variance between rats was of the same order as that for the lower degrees of pain in man.

It has been demonstrated by Miller (1948) and by Ipsen (1949) that there is little or no correlation between individual thresholds to heat stimulation before and after an analgesic. We have confirmed this observation with pressure stimulation, and have shown that no correlation should be expected, since the variance between repeated readings is as great as that between rats. This would indicate a high degree of homogeneity among the rats studied ; under such conditions the procedure of determining individual increases in pain threshold cannot therefore be recommended, as it only serves to increase the error of the test.

Miller (1948) in his *Critique of Analgesic Testing Methods* pointed out that no method had been evolved for combining into a single parameter measurements of the intensity of effect and duration of action of analgesics. While duration of action should be considered in assessing the value of new analgesic compounds, the combination of it with degree might result in important differences between rates of onset and duration of effect being overlooked. Estimates of the degree of analgesia are frequently invalidated by some animals in a group failing to respond to stimulation,

and in addition some practical considerations also make it undesirable to include duration of action among the routine observations. First, the time taken for several readings at intervals after injection reduces the number of animals which may be tested on any one occasion. Second, repeated subjection to high levels of pressure or long exposure to heat reduces the apparent duration of action of the drug. This may be overcome by the use of fresh animals for each time reading, but this is uneconomic. Further, from some small experiments we have carried out measuring the proportion of animals whose thresholds significantly exceeded the normal range for $p=0.99$, it would appear that the measurement of duration of action of analgesics is subject to wider experimental errors than the simple measurement of degree ; it is most likely therefore that a combination of the two would give less accurate results than degree alone. On these grounds we prefer to treat intensity of effects at an arbitrary time interval and duration as separate entities both in the design of the tests for determining them and in their interpretation.

When tested on rats by either the radiant heat or the pressure method the potency of amidone is constant in many age groups at about 2.3 times that of morphine. In contrast pethidine shows a more shallow regression than morphine and the relative activities of the two compounds vary with the level of analgesic effect. The difference in regression is probably largely responsible for the variability in the pethidine/morphine ratio observed in these laboratories and elsewhere. A more important difference is inherent in the observation that, relative to morphine, pethidine is about 1.5 times more active by the pressure method than by the radiant heat method. It seems that pethidine may have either a greater analgesic effect on pain caused by pressure than pain due to heat or a greater influence on the flight reaction to pain (struggle and squeak) than on the reflex of removing an appendage from a source of stimulus.

The example of pethidine emphasizes the necessity for considering the method of test when comparing analgesic ratios in the same species. It also shows that even within one species and with one method the relative activities of analgesics obtained may depend on the response level chosen and on the sensitivity of the animals used. The interval between injection and the measurement of thresholds may also be of importance ; in the comparative tests described above observations were made 30 minutes after subcutaneous injection.

Summary

1. A method is described for determining the pain threshold of rats to a gradually increasing pressure on the tail. These estimates and heat reaction times are log-normally distributed. The effect of age, sex, and site of stimulation were investigated. By both methods the variance between repeated readings is as great as between rats ; the importance of this in the design of analgesiometric tests is discussed.

2. The sensitivity of rats to morphine and amidone decreases up to a certain age. In an extensive comparison in rats of many age groups the ratio of the activity of amidone to morphine sulphate was constant at about 2.29 : 1 whether tested by the radiant heat or the pressure method. The fiducial limits of the ratio, for $p=0.95$, were 2.17 to 2.41.

3. The increase in the analgesic effect for a given increment in dose was less with pethidine than with morphine. For this reason the relative activity of the two compounds by any one method is dependent on the response level chosen and the sensitivity of the rats used. A comparison by the heat and pressure methods showed that pethidine had a relatively greater effect than morphine on the " flight reaction " in response to pressure on the tail of the rat than on the reflex of removing the tail from a source of radiant heat stimulation.

4. A small degree of " tolerance " to amidone and morphine occurred even after a single injection in the rat.

We wish to thank Dr. A. C. White and our assistants, Messrs. J. B. Attlesey, N. W. Dolling, G. Downs, and B. Kent, for their help. We are also indebted to Dr. Walker, of the Glaxo Laboratories, for the phenadoxone used in this investigation.

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APPENDIX

THE MECHANICAL DRIVE FOR THE PRESSURE APPARATUS

BY

E. I. GODFREY

The driving mechanism photographed in use in Fig. 1 is shown in more detail in Fig. 5. It consists of three main components mounted on a common base (D).

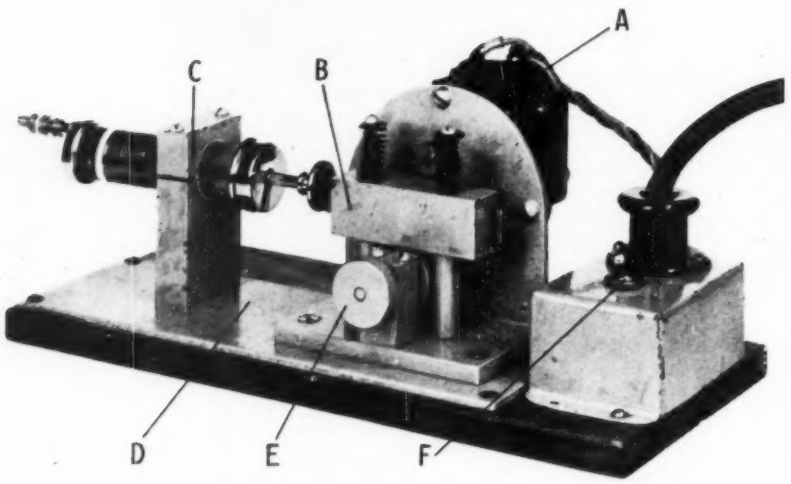

FIG. 5.—The mechanical drive of the pressure apparatus (for key see text).

These are the driving motor A, a "Synclock" electric motor (1 r.p.m.), suitably geared to a rack carried by the rack-casing B, and the syringe housing C in which is clamped the horizontal syringe of the pressure apparatus. E indicates a knob operating the quick-release mechanism described below, and F a switch controlling the motor.

The rack and its casing are removed for the photograph in Fig. 6. The gear G is mounted on the motor driving shaft, and operates on the coaxial gears H and I. The latter meshes with the rack when in operation. The knob E, operating the cam K, causes the rack casing to be raised or lowered on its supporting pillars J. In Fig. 7 the rack L may be seen in its casing B. The springs shown are to prevent the rack from lifting during the driving operation. When the rack is disengaged it is free to slide in its case, and the pressure in the syringe system is automatically released.

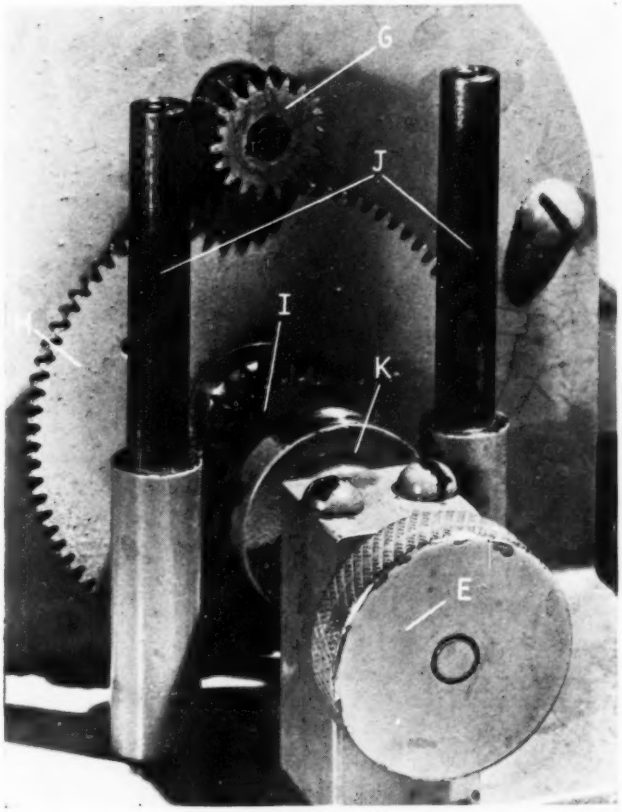

FIG. 6.—Details of the driving gears and quick-release device (rack and casing removed).

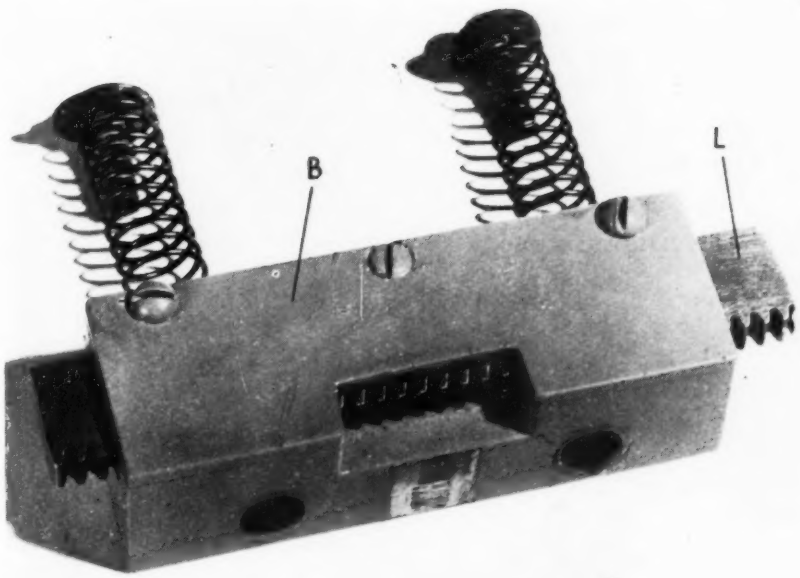

FIG. 7.—The rack and its casing.

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A STUDY OF THE ACTION OF ANTIMONIAL COMPOUNDS ON THE LIVER FLUKE (*FASCIOLA HEPATICA*) *IN VITRO*

BY

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(Received May 18, 1951)

Chance and Mansour (1949) showed that the liver fluke (*Fasciola hepatica*) could be used *in vitro* as a preparation for testing the action of anthelmintics. This made it possible to compare the direct action of established anthelmintics on a representative of the platyhelminths as well as on a nematode, since a similar study has been made by Baldwin (1943) on *Ascaris in vitro*. This comparison is an essential part of studies of the mode of action of anthelmintics, and has revealed important differences between those representatives of the two phyla in their response to the same drugs. With a few notable exceptions anthelmintics have a relatively low activity on the parasites themselves ; e.g., they do not compare in potency with antibacterials. Moreover, it has been found that anthelmintic activity does not always depend upon a lethal action, but may depend upon an interference with the movements of the parasite. This can happen in more than one way. Studies on the parasite *in vitro* are therefore valuable ways of increasing our knowledge of the action of these drugs. Chance and Mansour (1949) tested anthelmintic drugs mainly used in therapeutics against intestinal parasites ; the present report is concerned with the lethal action of the chief drugs used against blood fluke infestations, namely organic antimonials. The results reveal that the preparation is sensitive to a wider range of drugs than had previously been suspected ; they also reveal some facts about the mode of action of the substances which act on blood flukes as well as on those active in the alimentary tract. Since the somatic helminths are bathed in blood or lymph during life, a comparison has been made between the action of these drugs with and without serum added to the saline medium used in the earlier study.

Materials and Methods

Numerous organic antimony preparations are used as therapeutic agents against somatic helminths. The following compounds were therefore tested:

(*a*) Potassium antimonyl tartrate (tartar emetic).

(*b*) Antimony pyrocatechol sodium disulphonate (fouadin).

(*c*) *p*-Aminophenyl stibinate of diethylamine (neostibosan).

Kymographic records of the *in vitro* movements of the whole fluke were obtained by the method described in the earlier publication (Chance and Mansour, 1949).

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The preparations from bovine flukes were found to give rhythmical movements either in Ringer solution alone or 50 per cent (v/v) bovine serum-Ringer solution at a *p*H of 7.4 [tartar emetic is not stable in more alkaline solutions (Oelkers, 1937)] for a period of at least two hours, and frequently as long as six hours. The method of dosage employed for the therapeutic use of these drugs against schistosomiasis suggests that the rate of action is slow. Therefore, in testing these anthelmintic drugs the period of application to the preparation was extended up to 90 minutes. After that time the degree of interference with rhythmical movement of the preparation was tested by the addition of 1 : 5,000 solution of amphetamine sulphate, which has a stimulant effect on normal flukes. At least four flukes were used in each test.

RESULTS

Responses in saline.—Tartar emetic, fouadin, and neostibosan were tested at 1 : 1,000 concentration for as long as 90 minutes. The three compounds failed to cause paralysis of rhythmical activity or to prevent the response to amphetamine (Figs. 1 and 2).

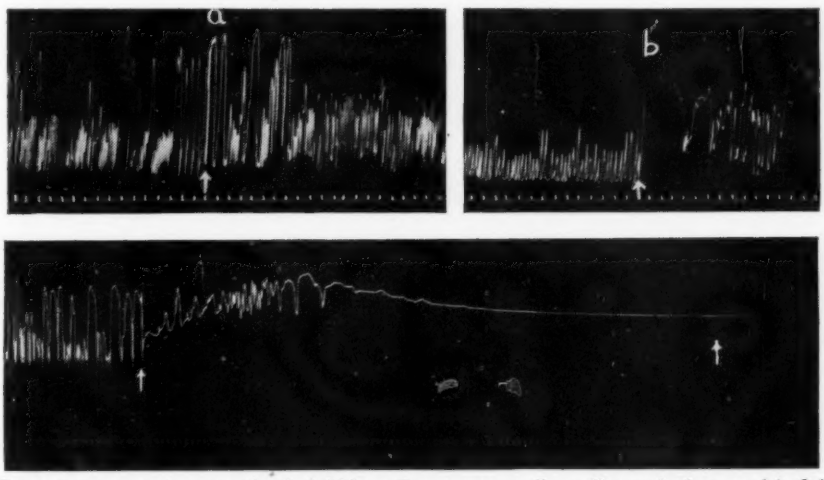

FIG. 1.—Responses to tartar emetic 1:1,000:—*Top tracing:* In saline solution at (*a*) followed by response to amphetamine after 90 minutes at (*b*). *Bottom tracing:* In serum saline medium followed by absence of response to amphetamine after 90 minutes. (In all tracings upward stroke represents contraction. Time markings in minutes.)

Responses in serum-saline.—In this medium, which was innocuous to the rhythmical activity of the preparation for as long as two hours, tartar emetic at a concentration of 1 : 1,000 caused a slight rise of tone in rhythmical movement which continued with smaller amplitude for 30 minutes (Fig. 1). Complete paralysis in the contracted form then occurred. No response to amphetamine was noticed after 90 minutes. The minimum amount of serum necessary to produce this potentiating effect was found to be 50 per cent in the saline medium. The minimal effective concentration for tartar emetic was 1 : 1,000. On the other hand, the presence of serum in the medium did not initiate any toxic response either to fouadin (Fig. 2) or to neostibosan up to a concentration of 1 : 1,000.

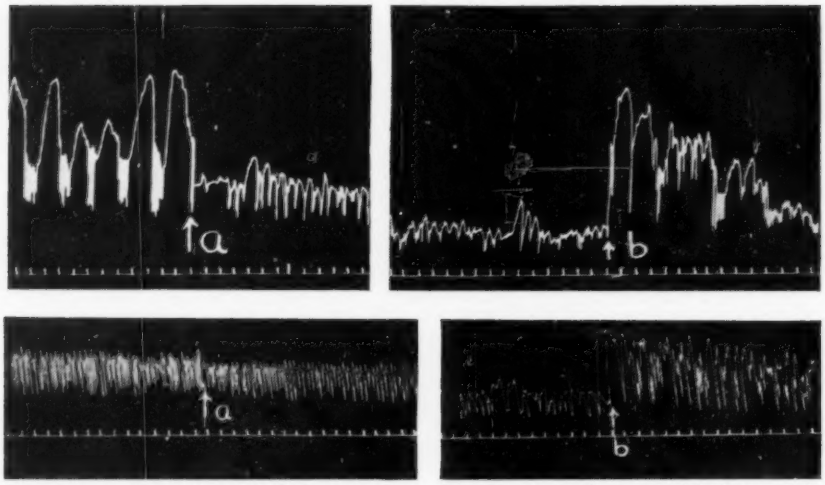

FIG. 2.—Responses to fouadin 1 : 1,000:—*Top tracing :* In saline solution at (*a*) followed by response to amphetamine after 90 minutes at (*b*). *Bottom tracing :* In serum saline medium at (*a*) followed by response to amphetamine after 90 minutes at (*b*).

Investigation of serum effect.—The potentiating effect of serum on tartar emetic drew attention to the fact that serum may play an important part in its parasiticidal action *in vivo* ; thus serum might stimulate the ingestion mechanism of the parasite *in vitro,* so that some of the drug would gain access through the gut, since serum is part of the main food for flukes (Stephenson, 1947). However, flukes with ligatured oral openings showed the same response as normal flukes to the toxicity of tartar emetic in serum.

Another factor which had to be considered was the presence of complement in the serum, but heating the serum for 30 minutes at 55° C. did not abolish its potentiating effect on the toxic action of tartar emetic.

The effect of dividing the serum into dialysable and non-dialysable fractions was also investigated.

Bovine serum (200 ml.) were dialysed in a cellophane sac against an equal volume of distilled water at 4° C. The distilled water was changed every 48 hours. Ten days' dialysate was collected, which was normally less than a litre in volume as the distilled water passed through the sac. On the other hand, the amount of serum was more than its original volume after this period of dialysis.

For kymographic tests, the amount of diluted serum, which originally contained 50 ml., was made up to 100 ml. with distilled water. Mineral content for 50 ml. of the saline medium was added to this amount ; 90 ml. of the dialysate was made up to 100 ml. with distilled water, with the addition of the mineral contents for 100 ml. of the original medium.

Neither dialysate nor serum affected the normal behaviour of movement when tested alone. Serum after dialysis was found to have lost the property of potentiating the activity of 1 : 1,000 tartar emetic (Fig. 3), whereas the dialysate possessed a partial potentiating action. When serum was dialysed against saline for a week, with a control dialysing against distilled water, it was found that the saline dialysate did not potentiate the effect of 1 : 1,000 tartar emetic, whereas the distilled water control was again active.

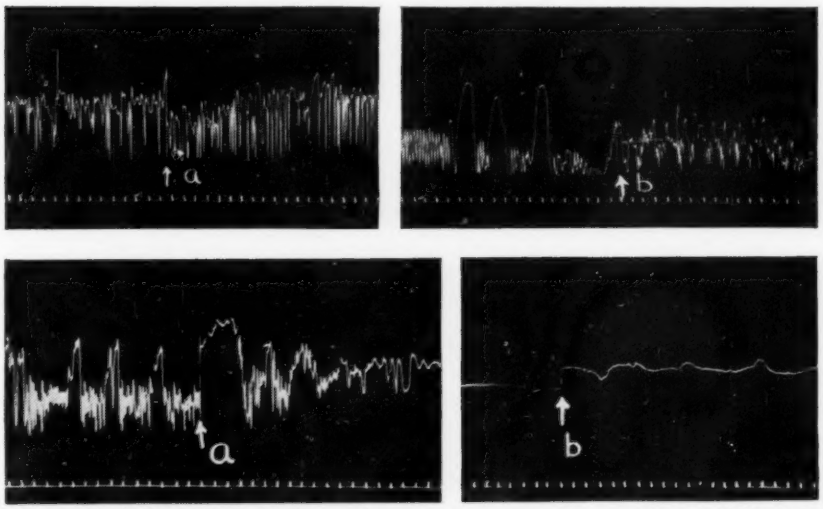

FIG. 3.—Effect of dialysis on potentiating action of serum on tartar emetic 1 : 1,000:—*Top tracing :* Effect of serum dialysed against distilled water for ten days on the action of tartar emetic at (*a*) followed by response to amphetamine after 90 minutes at (*b*). *Bottom tracing :* Effect of the freeze-dried dialysate on the action of tartar emetic at (*a*) followed by absence of full response to amphetamine after 90 minutes at (*b*).

Part of the distilled water dialysate from the previous experiment was freeze dried in order to test it in a more concentrated form. The amount of the freeze-dried powder from about 200 ml. dialysate corresponded to the total dialysate from 50 ml. bovine serum ; it was dissolved in 50 ml. distilled water and made up to 100 ml. with Ringer solution, the *p*H being adjusted to 7.4. This solution was found to potentiate the lethal action of 1 : 1,000 tartar emetic on the preparation (Fig. 3).

A petroleum-ether-soluble fraction of the freeze-dried dialysate had no potentiating effect on the toxicity of tartar emetic. On the other hand, the residual water-soluble fraction was found to potentiate the effect of tartar emetic to some extent.

DISCUSSION

The results reported here were obtained by experiments which lasted for 90 minutes. The compounds tested did not show any anthelmintic activity in the saline medium. On the other hand, a definite lethal action was demonstrated to tartar emetic in the presence of serum. This fact indicates the possible importance of the environment of the parasite on the effect of anthelmintic drugs. Lièvre (1934) reported an unsuccessful attempt to demonstrate the lethal action of tartar emetic on the liver fluke. It seems likely that this was due to the fact that a saline medium was used.

Although the antimony in fouadin is in the trivalent state, as in tartar emetic, fouadin failed to paralyse the preparations in serum saline. This might be due to its low antimony content. However, neostibosan was inactive in spite of its high antimony content. The low lethal activity of the pentavalent antimony compound, neostibosan, *in vitro* was demonstrated by Lee and Chung (1935), who found that *Schistosoma japonicum* in serum cultures was not killed at a concentration of 1 : 6,000, except after eight days.

The potentiating action of serum on tartar emetic is not due to complement and it is retained in the dialysable fraction.

Summary

1. The effects of antimonial compounds on preparations of the liver fluke (*Fasciola hepatica*) have been recorded kymographically. Both Ringer's solution and a mixture of equal volumes of bovine serum and Ringer's solution were used as media for these *in vitro* tests for a maximum period of 90 minutes.

2. Tartar emetic, fouadin, and neostibosan all failed to cause paralysis of rhythmical movement in a saline medium in a concentration of 1 : 1,000.

3. Serum was found to initiate a lethal response to tartar emetic, but not to fouadin or neostibosan. The fraction of serum responsible for this action was found to be dialysable through cellophane membrane against distilled water, but not against saline.

It is a pleasure to express my indebtedness to Professor A. C. Frazer and Dr. M. R. A. Chance for continued guidance and stimulating interest; the Veterinary Department of the City of Birmingham Meat Market for their helpful co-operation; and Mr. G. A. Rowe for preparing photographs of kymographic records. My thanks are also due to Dr. M. L. Tainter, of the Sterling-Winthrop Research Institute, for the supply of fouadin and neostibosan.

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THE INHIBITION OF CHOLINESTERASES BY ALKYLPHOSPHATES AND ALKYLPHENOLPHOSPHATES

BY

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(Received May 31, 1951)

The study of anticholinesterases belonging to the group of alkylphosphates has presented some new and interesting kinetic problems. The inhibition of cholinesterase by eserine, neostigmine, and related compounds is due to the formation of a reversible complex between the enzyme and the entire inhibitor molecule (Aeschlimann and Reinert, 1931 ; Bloch, 1939 ; Randall and Lehmann, 1950). The duration of action *in vivo* of anticholinesterases of this type depends on the rate of dissociation of the enzyme-inhibitor complex and the rate at which the free inhibitor is removed from the body by metabolism and excretion.

Another type of cholinesterase inhibition has been found with a group of compounds derived from dialkylphosphoric esters. Evidence has been adduced to show that these alkylphosphates inhibit by phosphorylation of the active enzyme centre of cholinesterase (Burgen, 1949b ; Wilson and Bergmann, 1950). This type of inhibition has commonly been called " irreversible " but closer study has shown marked differences in the duration of action between different members of this group (Grob, 1950 ; Hobbiger, 1951). If alkylphosphates inhibit cholinesterase by means of a phosphorylation process, the stability of the enzyme-inhibitor complex should be the same whenever the same alkylphosphate group is transferred, or in other words the nature of the alkyl group should be the determining factor in the rate of enzyme recovery. This problem can be studied by using alkylphosphates of a homologous series, where the only variable is the alkyl group. We have studied the behaviour of a series of such compounds, which combined the aminophenol feature of neostigmine and the dialkylphosphoryl group of tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP). In the account that follows the kinetics of cholinesterase inhibition by these compounds are described and correlated with those of neostigmine, TEPP, and DFP. Investigations of their action *in vivo* are also presented.

Methods and Materials

Fresh oxalated human plasma and washed human red cells, lysed by the addition of four volumes of 0.025 M-$NaHCO_3$, were used as a source of cholinesterase II (pseudo cholinesterase) and cholinesterase I (true cholinesterase) respectively. The activity of the enzyme was estimated by the Warburg technique (Ammon, 1933) at 37° C. with 0.025 M-$NaHCO_3$ as medium, equilibrated with a 95 per cent N_2 + 5 per cent CO_2 gas mixture. The enzyme solution was placed in the main compartment

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of the vessel, and substrate and inhibitor, each dissolved in 0.2 ml. 0.025 M-$NaHCO_3$, were placed in separate sidearms. The total volume of fluid was always 3 ml.; *dl*-acetyl-β-methylcholine (final concentration 0.03 M) and benzoylcholine (final concentration 0.01 M) were used as substrates for cholinesterases I and II respectively. Corrections for non-enzymic hydrolysis of the substrates were applied in all experiments.

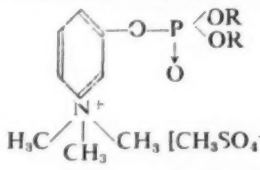
In order to study the recovery of cholinesterase activity *in vitro* human red cell esterase was used ; the details of the experimental procedure are given in the text.

The duration of action *in vivo* was studied in rats and rabbits. In rats the chromodacryorrhoea response, as described by Burgen (1949a), was used as an overall indicator of the duration of enzyme inhibition.

The duration of action of anticholinesterases on striated muscle was studied on the isolated phrenic nerve-diaphragm of the rat (Bülbring, 1946). The diaphragm was suspended in 100 ml. Tyrode solution (containing 0.2 per cent (w/v) glucose) aerated with 95 per cent $O_2 + 5$ per cent CO_2. The phrenic nerve was stimulated through submerged electrodes with slightly supramaximal square pulses of 0.25 msec. duration from an electronic stimulator. A spring-loaded lever was used for recording the contractions. After the diaphragm had been set up for 30 minutes the anticholinesterases were added to the bath for one hour, during which time a continuous stimulation at a frequency of 3 stimuli per minute was applied to the nerve. After the preparation had been in contact with the anticholinesterase for an hour 3 bursts of tetanic stimulation (25, 50, and 100 stimuli per second), each lasting 5 seconds, were given at 30-second intervals. The bath fluid was now replaced by fresh Tyrode solution (without anticholinesterase) and later again every 10 minutes. The response of the diaphragm to tetanic stimulation was tested each time before replacement of the bath fluid.

The distribution of the anticholinesterases *in vivo* was studied in male albino wistar rats of 150 to 200 g. weight, and details of the experimental procedure are given in the text.

The compounds studied had the following general chemical structure:



Code No.	R	Chemical name	Short title
Ro 3-0412 ..	$-CH_3$	*m*-(dimethylphosphato)-N-trimethylanilinium methylsulphate	Dimethylester
Ro 3-0340 ..	$-CH_2CH_3$	*m*-(diethylphosphato)-N-trimethylanilinium methylsulphate	Diethylester
Ro 3-0411 ..	$-CH(CH_3)_2$	*m*-(di*iso*propylphosphato)-N-trimethylanilinium methylsulphate	Di*iso*propylester
Ro 3-0397 ..	$-CH(CH_3)(CH_2CH_3)$	*m*-(di*sec*butylphosphato)-N-trimethylanilinium methylsulphate	Di*sec*butylester

All these compounds, which are referred to in the text by the above-mentioned short titles, or in general as "dialkylphosphostigmines," were synthesized in the Research Department of Roche Products, Ltd.* They were made available as pure crystalline

* The chemistry of these compounds will be published elsewhere.

substances readily soluble in water. Fresh aqueous solutions were always made before use.

TEPP and DFP were made up as 1 per cent (w/v) stock solutions in dry propylene glycol and dilute aqueous solutions were made from these immediately before use.

Neostigmine was used as the methylsulphate.

RESULTS

Inhibition of cholinesterase in vitro

In these experiments the inhibitor was added at room temperature to the enzyme solution, which was incubated at 37° C. for 20 minutes. The substrate was then added and the total CO_2 output between 5 and 35 minutes after addition of the substrate was taken as the measure of enzyme activity.

TABLE I

INHIBITION OF CHOLINESTERASE *in vitro* BY NEOSTIGMINE, TEPP, AND DIALKYLPHOSPHOSTIGMINES

Inhibitor	Molar concentration required for 50% inhibition of		Ratio $\frac{A}{B}$
	Red cell esterase A	Serum esterase B	
Neostigmine	1.14×10^{-7}	6.9×10^{-7}	0.17
TEPP	1.2×10^{-8}	8×10^{-10}	15
Dialkylphosphostigmines:			
Dimethyl-	7.5×10^{-8}	6.3×10^{-9}	12
Diethyl-	8×10^{-8}	1.2×10^{-9}	66
Di*iso*propyl-	2.1×10^{-7}	3×10^{-9}	70
Di*sec*butyl-	1.6×10^{-7}	9×10^{-9}	18

It will be seen from Table I that the dialkylphosphostigmines are powerful inhibitors of cholinesterase *in vitro*. Their potency compares favourably with that of neostigmine and TEPP. All four esters are more potent inhibitors of serum (Ch.E. II) than of red cell esterase (Ch.E. I), and the difference is especially marked with the diethyl- and di*iso*propyl-esters, which inhibit serum esterase by 50 per cent at dilutions respectively 66 and 70 times greater than those required to produce the same degree of inhibition of red cell esterase.

Kinetics of the cholinesterase-inhibitor complex

A closer study of the time course of inhibition and the kinetics of the dissociation of the cholinesterase-inhibitor complex showed that the inhibition of cholinesterase by dialkylphosphostigmines proceeds at a rather slow rate, and with a given concentration of inhibitor a maximal inhibition is not reached even after two hours, except with the dimethylester which produces a maximal inhibition, for a given concentration, within an hour.

It is not possible to make an accurate comparison of the time course of inhibition produced by dialkylphosphostigmines with that produced by TEPP and DFP, since the two latter compounds are much less stable in aqueous solutions (Table II).

The kinetics of inhibition differ according to the ester used, and generally we can divide the dialkylphosphostigmines into two main groups:

TABLE II

NON-ENZYMATIC HYDROLYSIS OF AQUEOUS SOLUTIONS OF TEPP, DFP, AND DIALKYLPHOSPHOSTIGMINES

Inhibitor	Initial concentration g./100 ml.	Times in hours required for 50% hydrolysis	Incubation temperature
DFP	1	7.0	37° C.
TEPP	1	3.5	37° C.
Dialkylphosphostigmines:			
Dimethyl-	1	200	45° C.
Diethyl-	1	430	45° C.
Di*iso*propyl-	1	750	45° C.
Di*sec*butyl-	0.01	180	37° C.

(a) The dimethylester.—When cholinesterase was first incubated with the dimethylester, and then the substrate added, the enzyme activity increased steadily during the first 30 minutes, by which time a state of equilibrium was reached (Fig. 1). The slope of the dose-response curve was similar to that obtained with a

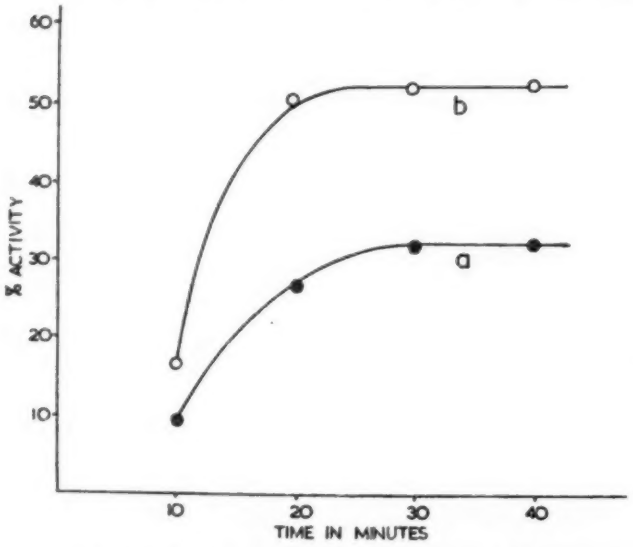


FIG. 1.—Human red cell esterase incubated for 20 min. with dimethylphosphostigmine in a molar concentration of 1.4×10^{-6} (*a*) and 1.5×10^{-7} (*b*): at zero time addition of 0.025 M-*dl*-acetyl-β-methylcholine as substrate. Ordinate : activity in per cent of uninhibited control for periods of 10 min. Abscissa: time in minutes after addition of substrate.

reversible inhibitor like neostigmine, and the concentration of the inhibitor had to be increased roughly 100 times in order to increase the inhibition from 30 to 70 per cent.

(b) The diethyl-, diisopropyl-, and disecbutyl-esters.—With these three esters addition of the substrate after the inhibitor had been in contact with the enzyme for 20 minutes merely prevented further enzyme inactivation, and no enzyme reactivation occurred. If the substrate was given before the inhibitor (the concentration of inhibitor being that which by itself caused 50 per cent reduction of cholinesterase activity in 20 minutes) no enzyme inactivation was seen during the next 40 minutes. This blocking effect by the substrate could be overcome with higher concentrations of the esters (Fig. 2). The slope of the dose-response curve was steep, and, on the

average, increasing the concentration of these esters that produced 30 per cent inhibition threefold raised the percentage inhibition of cholinesterase to 70 per cent. These results are identical with those found with TEPP and DFP (Burgen, 1949b).

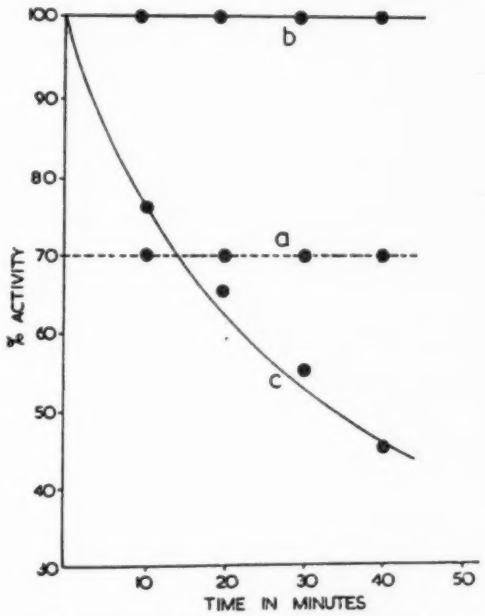


FIG. 2.—Inhibition of human red cell esterase by diethylphosphostigmine. Ordinate: activity as percentage of uninhibited control. Abscissa: time in minutes. Substrate: *dl*-acetyl-β-methylcholine. In (*a*) the enzyme was incubated with 5×10^{-8} M-diethylester for 20 min. before addition of the substrate. In (*b*) the diethylester (final conc. 5×10^{-8} M) was added 5 min. after the substrate. In (*c*) the diethylester (final concentration 5×10^{-6} M) was added 5 min. after the substrate.

It has been shown that cholinesterase previously inactivated by TEPP slowly regains its activity *in vitro* as soon as hydrolysis of the uncombined inhibitor molecules has occurred; this is not true with cholinesterase inhibited by DFP (Hobbiger, 1951). Experiments of this type were repeated with cholinesterase inhibited by dialkylphosphostigmines, the following technique being used:

Intact human red cells were suspended in a 0.9 per cent NaCl solution containing the inhibitor, and kept at room temperature for 30 minutes. The excess uncombined inhibitor was then removed by centrifugation and repeated washing of the red cells with fresh saline; 1 ml. packed red cells was then haemolysed in 49 ml. 0.025 M-$NaHCO_3$ and the haemolysate incubated at 37° C. The cholinesterase activity was measured shortly after haemolysis, and again after incubation for 24 and 48 hours.

Cholinesterase, which had been inactivated in this way by the diethylester, showed reactivation *in vitro* after removal of the uncombined inhibitor. The time course of enzyme recovery was similar to the time course of enzyme recovery after TEPP (Table III). This recovery was a true reactivation and was not due to bacterial action, since bacteriostatic agents did not influence it significantly.

When cholinesterase was inhibited by the di*iso*propyl or di*sec*butyl ester no reactivation of the enzyme was found *in vitro*. These esters behave therefore like DFP (Table III).

The duration of the actions of neostigmine, TEPP, DFP, and dialkylphosphostigmines on the isolated rat phrenic nerve diaphragm preparation

In Fig. 3 the responses to tetanic stimulation at 25, 50, and 100 stimuli/sec. are shown. The normal type response is seen in Fig. 3*a*(C). It can be seen from Fig. 3

TABLE III

TIME COURSE OF REACTIVATION OF CHOLINESTERASE I *in vitro* AFTER REMOVAL OF THE UNCOMBINED INHIBITOR AT 37° C.

Inhibitor	After removal of uncombined inhibitor		After 24 hours		After 48 hours	
	μl. CO_2 output/10 min.	Activity in % of control	μl. CO_2 output/10 min.	Activity in % of control	μl. CO_2 output/10 min.	Activity in % of control
(1) Control ..	52.5	100	46.2	88	44.7	85
+ TEPP (10^{-6}M)	1.9	4	18.1	41	23.3	52
+ Diethylphosphostigmine (10^{-5}M).. ..	10.0	18	19.7	42	23.0	51
(2) Control ..	39.5	100	36.9	93	37.5	95
+ TEPP (10^{-6}M)	3.3	8	11.9	32	18.0	48
+ Diethylphosphostigmine (10^{-5}M).. ..	6.3	15	11.2	31	17.9	47
(3) Control ..	56.6	100	51.8	92	38.8	70
+ DFP (10^{-5}M)	4.8	9	1.3	3	1.3	4
+ Di*iso*propylphosphostigmine (10^{-5}M) ..	7.1	12	4.5	9	3.2	8

that the response to a tetanus was always reduced to a single twitch after the preparation had been in contact with an anticholinesterase, whereas in a normal preparation a tetanus was always fully maintained. Fig. 3*a* illustrates that the effect produced by neostigmine is readily reversible and that 30 minutes after removal of the inhibitor a tetanus is again fully maintained with all three frequencies of stimulation. In the experiments with dimethylphosphostigmine (Fig. 3*b*) this recovery was somewhat slower, but occurred, as with neostigmine, with all three frequencies. Diethylphosphostigmine, however, had a much longer duration of action (Fig. 3*d*), and no recovery of the tetanic response to a stimulation with 100 stimuli/sec. could be seen within 90 minutes after removal of the inhibitor, by which time the response to a lower rate of stimulation (50 stimuli/sec.) was still less than it was 30 minutes after the removal of neostigmine or dimethylphosphostigmine. TEPP (Fig. 3*c*) behaved exactly like diethylphosphostigmine. With DFP and di*sec*butylphosphostigmine no significant recovery of the response to tetanic stimulation with 50 stimuli/sec. took place within 90 minutes after removal of the inhibitor. With di*iso*propylphosphostigmine the recovery for the same frequency of stimulation is somewhat more marked, but still much less than with TEPP. No recovery was seen for the three inhibitors with higher rates of stimulation.

Duration of action in vivo

The duration of action *in vivo* was studied in rats, the chromodacryorrhoea response as described by Burgen (1949a) being used. In this test the amount of acetylcholine required to produce the secretion of red tears is a measure of cholinesterase activity. After an injection of an anticholinesterase the threshold for

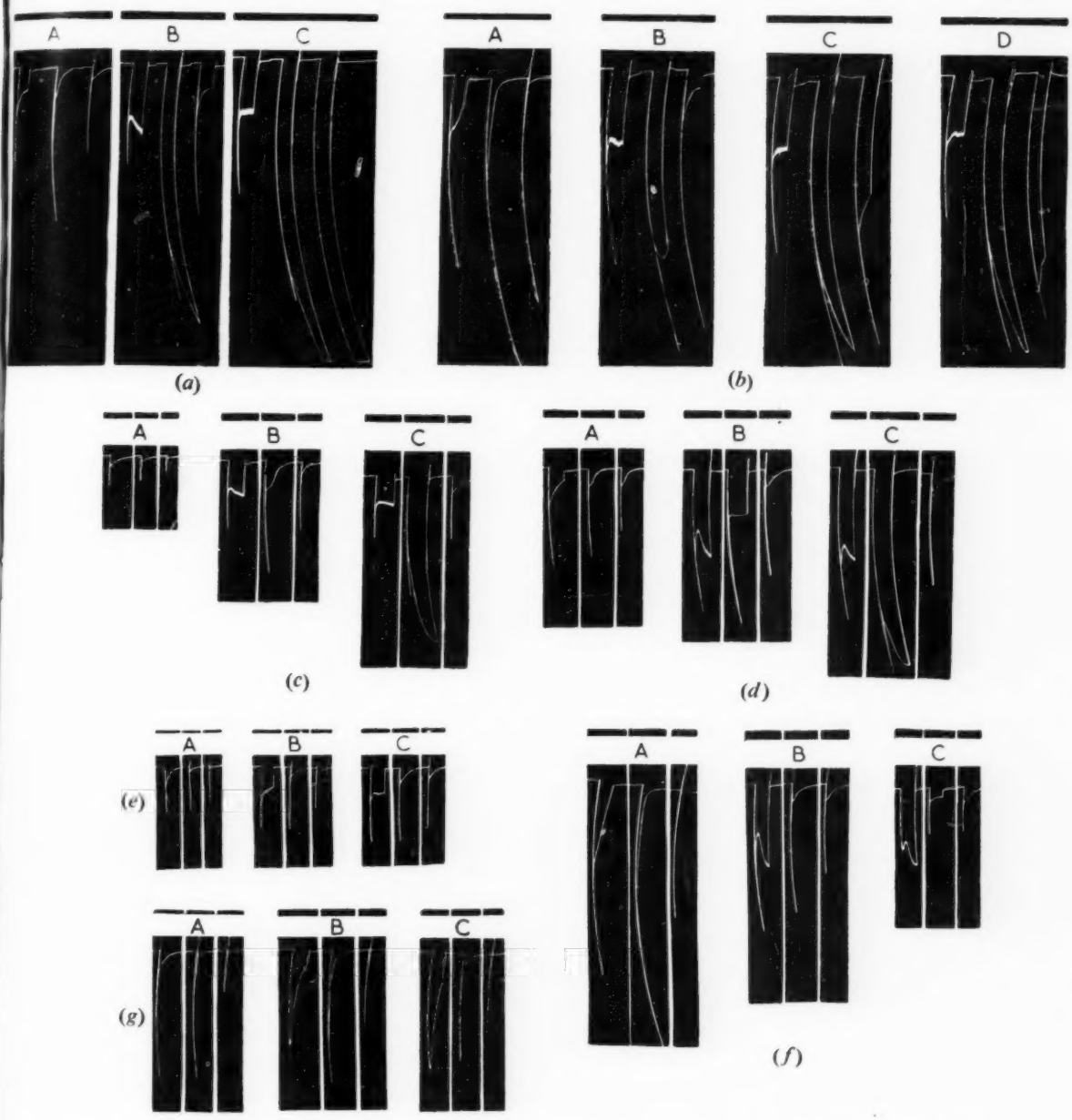


FIG. 3.—Rat phrenic nerve diaphragm (see text). Responses to a 5 sec. tetanus of 25, 50, and 100 stimuli/sec. lasting for 5 sec. (*a*) A: in presence of 0.2 mg. neostigmine methylsulphate/100 ml., left in contact with the diaphragm for 1 hr. previously. B: 10 min., and C: 30 min. after removal of the inhibitor. (*b*) A: in presence of 2 mg. dimethylphosphostigmine/100 ml. left in contact with the diaphragm for 1 hr. previously. B: 10 min., C: 20 min., and D: 40 min. after removal of the inhibitor. (*c*)–(*g*) Responses (A) 10 min., (B) 50 min., and (C) 90 min. after removal of the inhibitor, which had been in contact with the diaphragm for 1 hr. The inhibitors were: (*c*) 2 mg. TEPP/100 ml. (*d*) 5 mg. diethylphosphostigmine/100 ml. (*e*) 2 mg. DFP/100 ml. (*f*) 5 mg. di*iso*propylphosphostigmine/100 ml. (*g*) 5 mg. di*sec*butylphosphostigmine/100 ml.

acetylcholine is lowered, and in our experiments we gave a dose of the inhibitor which produced an increase in the sensitivity of about tenfold in each experiment. With neostigmine the acetylcholine threshold returned to normal on the second

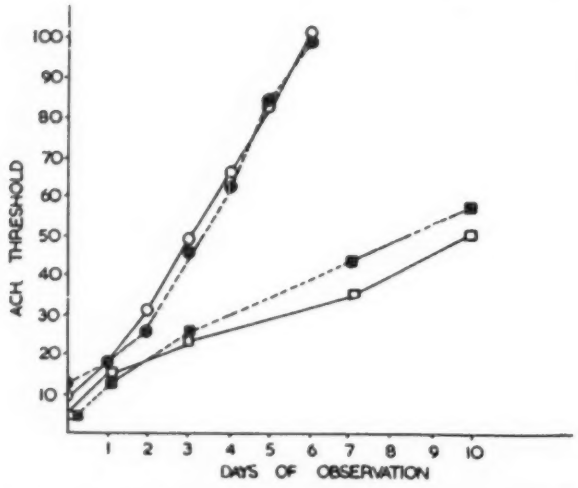


FIG. 4.—Time course of recovery of cholinesterase activity in rats, tested by the chromodacryorrhoea response. Ordinate: amount of acetylcholine (as % of 3 tests before injection of inhibitor) which had to be given to stimulate the secretion of red tears. Abscissa: days of observation. O———O: TEPP (0.4 mg./kg.). □———□ DFP (4 mg./kg.). ●- - -●: diethylester (3 mg./kg.). ■- - -■ di*iso*propylester (13 mg./kg.).

day; the action of the dimethylester wore off in four days; the diethylester and TEPP acted for about seven days. With DFP, the di*iso*propyl- and di*sec*butylesters, which all behaved very similarly, even after 10 days the rats were still twice as sensitive to acetylcholine as before the injection of the inhibitor (Fig. 4).

In rabbits the recovery of red cell esterase was studied after intravenous injection of the inhibitors. Here again the dimethylester had the briefest action of the dialkylphosphostigmines, the diethylester very closely resembled TEPP, and the di*iso*propylester behaved like DFP.

Inhibition of cholinesterase by dialkylphosphostigmines in blood and tissues in vivo

Parenteral administration of dialkylphosphostigmines in rats produced symptoms of intoxication which were closely correlated with the degree of inhibition of cholinesterase.

Injection of the dimethylester caused the appearance of muscarinic and nicotinic symptoms of acetylcholine poisoning, closely resembling those seen after the injection of neostigmine. The diethyl-, di*iso*propyl-, and di*sec*butyl-esters, in doses which had no effect on striated muscle, produced marked symptoms of the muscarinic type, e.g., diarrhoea, lacrimation, salivation, and bradycardia. In higher doses they also caused twitching and fibrillation, and at the same time the muscarinic symptoms became very pronounced.

The inhibition of cholinesterase in blood and tissues *in vivo* was studied in rats which received a subcutaneous injection of various doses of the diethyl- and di*iso*propyl-esters. The animals were killed 30 minutes after the injection of the inhibitor, and the cholinesterase activities of the blood, Harderian glands, intestine, and brain were estimated by the usual Warburg technique. Blood was removed from the three latter tissues by repeated washings and the tissues were then homogenized. As will be seen from Fig. 5, a concentration of the inhibitor which produced a reduction of

50 per cent in the esterase activity of red cells and serum did not significantly affect the cholinesterase activity in brain, Harderian glands, and intestine. Higher concentrations of the inhibitor, which reduced the activity of the esterases in blood by 80 per cent, caused a similar degree of enzyme inhibition in Harderian glands and intestine, but had no effect on the cholinesterase in brain. TEPP and DFP, on the other hand, given in a dose which inhibited red cell esterase by 80 per cent, reduced the activity of brain cholinesterase by 50 and 90 per cent respectively.

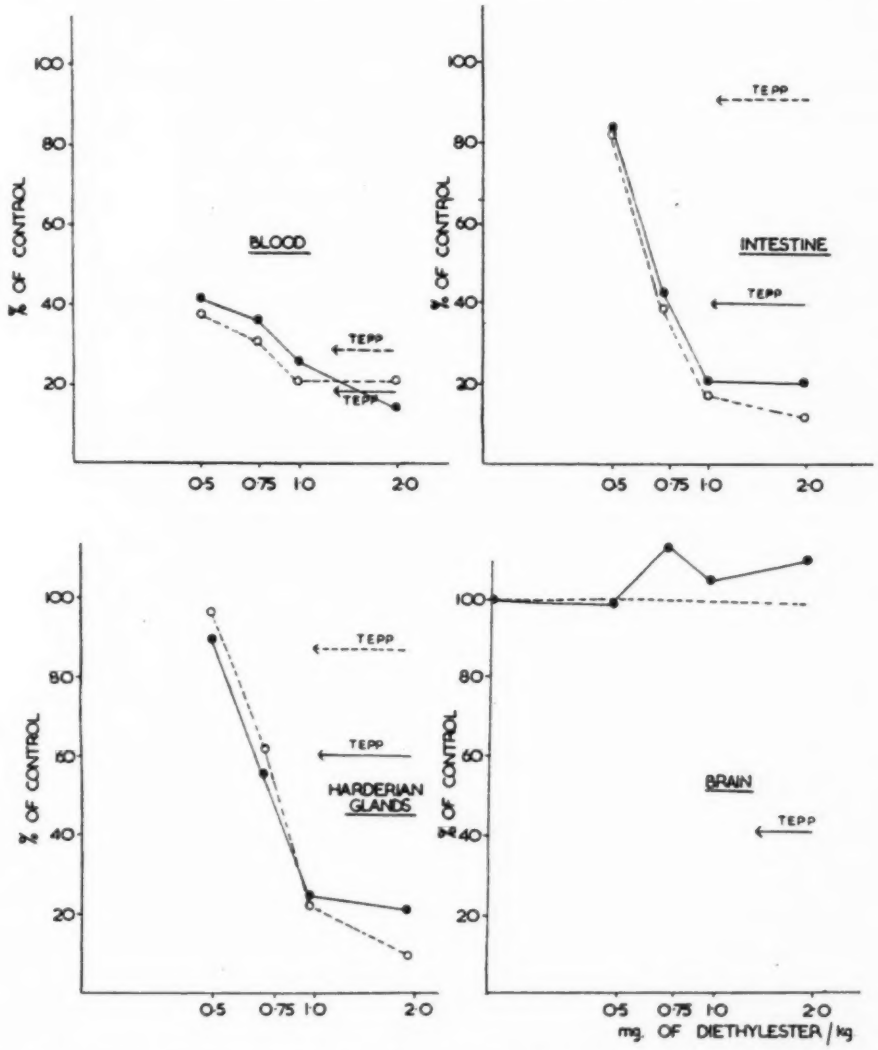


FIG. 5.—Inhibition of cholinesterase in rats *in vivo* by diethylphosphostigmine. Ordinates: cholinesterase activity expressed as a percentage of untreated controls. Abscissae: dose of the diethylester in mg./kg. The inhibitor was given by s.c. injection 30 min. after the injection of 2 mg. atropine/kg. The rats were killed 30 min. after the injection of the diethylester. The arrows on the right side give the degree of inhibition produced by 0.2 mg. TEPP/kg. ●———● cholinesterase I, O- - -O cholinesterase II.

Toxicities of dialkylphosphostigmines, neostigmine, TEPP, and DFP

Acute toxicities were determined in white mice, weighing 15–20 grammes, by the intravenous route. As can be seen from Table IV, the dialkylphosphostigmines

TABLE IV

	Mouse LD50 by intravenous injection in mg./kg.	Mouse LD50 $1{,}000 \times I_{50}$ *in vitro* (Ch.E. I)
Neostigmine	0.30	2.6
TEPP	0.18	8.0
DFP	0.4	1.7
Dialkylphosphostigmines :		
Dimethyl-	0.57	7.6
Diethyl-	7.5	94
Di*iso*propyl	25	120
Di*sec*butyl-	12.8	80

are, with the exception of the dimethylester, much less toxic than neostigmine, TEPP and DFP. The ratio of the mouse LD50 to the amount producing 50 per cent inhibition of the enzyme *in vitro* (I_{50}) is reasonably constant for the diethyl-, di*iso*propyl-, and di*sec*butyl-esters at levels far above those for the other compounds examined. On the other hand, the ratio for the dimethylester is not markedly different from that for neostigmine or TEPP. The remarkably low toxicity of the first-named esters is surprising and cannot be adequately explained at the present time.

Discussion

The inhibition of cholinesterase by TEPP and DFP was interpreted as a phosphorylation process by Burgen (1949b). Later Wilson and Bergmann (1950), studying the inhibition produced by alkylphosphates in relation to the *p*H of the incubation medium, came to the same conclusion. However, owing to the lack of a crystalline cholinesterase preparation no study as conclusive as that of Jansen *et al.* (1949 ; 1950) on α-chymotrypsin inhibition by DFP has yet been carried out. These authors found that 1 mol DFP combined with 1 mol chymotrypsin and at the same time 1 mol free HF appeared in solution. It is very likely that a similar process occurs with cholinesterase. There is good evidence that the phosphorylation of the enzyme is preceded by an initial attachment of the inhibitor to the enzyme. Once phosphorylation has occurred reactivation of cholinesterase could be brought about by an uncatalysed or enzymic hydrolysis. The kinetics of these reactions can be presented in the following way:

$$[E] + [I_{a+b}] \underset{\leftarrow}{\overset{K_1}{\rightarrow}} [E][I_{a+b}] \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

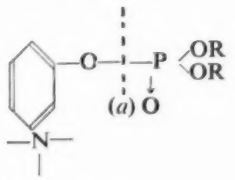
$$[E][I_{a+b}] \xrightarrow{K_2} [EI_a] + [I_b.H] \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$[EI_a] + [H_2O] \xrightarrow{K_3} [E.H] + [I_aOH] \quad \ldots\ldots\ldots (3)$$

where E = enzyme, I_{a+b} = whole inhibitor, I_a = dialkylphosphoryl moiety of the inhibitor, I_b = residue of the inhibitor molecule.

The velocities and equilibrium points of the above three reactions are determined by the different features of the structure of the inhibitor molecule. Reaction (1) depends on the general fit and bonding properties of the inhibitor molecule. With reversible inhibitors of the eserine and neostigmine type this is the only reaction involved, and it would be expected that the closer the structural resemblance of a phosphorylester to these inhibitors, or the enzyme substrates, the further equilibrium (1) will be shifted to the right.

The velocity of process (2) will depend on the initial lability of the ester linkage in the molecule, i.e., in the compounds described here most probably in bond (*a*):



It will thus be a function of both the left-hand part of the molecule and the nature of the alkyl groups.

The velocity of process (3), on the other hand, will depend on the stability of the EI_a complex. Assuming that enzymic breakdown of this complex does not occur (Hobbiger, 1951) the stability relations can be predicted in a more or less satisfactory manner from the physicochemical properties of the alkylester group. A greater stability of the EI_a complex would be expected with (i) increasing length of the alkyl chain and (ii) with a branched alkyl chain. It would seem that the only other important factor involved in this reaction in the body is the *p*H of the enzyme environment ; this might cause differences in the duration of inhibition in various tissues.

To date the only relevant reference to the duration of action in a homologous series is that by Saunders and Stacey (1948), who found that the miotic action of dimethyl- and diethyl-fluorophosphonates was briefer than that of DFP.

In the experiments reported here strong evidence is given to support the theory that the dialkylphosphoryl residue of the inhibitor molecule determines the duration of action. The duration of action of diethylphosphostigmine both *in vitro* and *in vivo* resembles the duration of action of TEPP, and similarly the duration of action of di*iso*propylphosphostigmine resembles that of DFP. The di*sec*butylphosphostigmine does not offer any further prolongation of action because the di*iso*propylphosphoryl-enzyme complex is already so stable that the rate of recovery is probably dependent on the rate of enzyme resynthesis (Koelle and Gilman, 1949).

It should be made clear that these kinetics only apply if the alkylphosphates produce their inhibition by phosphorylation. The evidence suggests that the brief duration of the action of dimethylphosphostigmine is due to failure of phosphorylation rather than to the formation of an unstable phosphorylated enzyme. This can be seen on three grounds: (1) The decrease of inhibition with addition of substrate, showing that a large part of the inhibition is competitive and reversible. (2) The fact that the inhibition reaches an equilibrium point and maintains it. If an unstable phosphorylated enzyme were formed this would mean a steady shift of the dimethylester through the three reactions to its breakdown products, and no equilibrium

would be reached. Fig. 1 shows that that is not so. (3) The degree of inhibition increases only slowly with increase of the inhibitor concentration; this is characteristic of the reversible type of cholinesterase inhibition. The kinetics of the dimethylphosphostigmines therefore resemble closely those of neostigmine and other reversible inhibitors.

Actions of dialkylphosphostigmines in vivo

In comparing the actions of various anticholinesterases *in vivo* we have to consider the following points: distribution via the blood stream, passage from blood to tissues, access to cholinesterase, kinetics of the cholinesterase inhibitor complex, etc. In spite of equal potency *in vitro* anticholinesterases can therefore very likely produce a different degree of enzyme inhibition in various cholinergically innervated structures *in vivo*; thus the brain is completely spared from inhibition by the dialkylphosphostigmines *in vivo*, even with lethal doses, but *in vitro* these esters are powerful inhibitors of brain cholinesterase; the *in vivo* findings can therefore only be explained by the action of the "blood-brain barrier" and the physicochemical properties of these anticholinesterases, which are lipoid insoluble compounds.

The diethyl- and di*iso*propyl-esters act preferentially on organs where acetylcholine exerts its muscarinic type of action. These actions are more marked than with TEPP or DFP. In previous work (Hobbiger, 1951) it was shown that TEPP, which *in vitro* inhibits Ch.E. II in concentrations one-fifteenth of those required to inhibit Ch.E. I, does not show such a preferential inhibition *in vivo*, and in the intestine even the reverse is the case. The diethyl- and di*iso*propyl-esters of the dialkylphosphostigmines, which are even more specific inhibitors of cholinesterase II *in vitro*, show the same lack of differentiation between the two enzymes *in vivo*, as can be seen from Fig. 5, and the muscarinic symptoms produced by them *in vitro* can be fully explained by the inhibition of true esterase.

Summary

1. The dimethyl-, diethyl-, di*iso*propyl-, and di*sec*butyl-esters of *m*-hydroxy-N-trimethylanilinium methylsulphate are potent inhibitors of cholinesterase.

2. *In vitro* they inhibit human red cell cholinesterase 50 per cent at concentrations ranging from 7.5×10^{-8} M to 1.6×10^{-7} M. Serum cholinesterase is inhibited by lower concentrations than red cell cholinesterase, especially by the diethyl- and di*iso*propyl-esters.

3. The dimethylester is a reversible inhibitor with properties similar to neostigmine, but with a longer duration of action.

4. The inhibition of cholinesterase by the diethylester is very similar to that produced by TEPP. With both compounds reactivation of the enzyme occurs slowly *in vitro*. The time courses of recovery of enzyme activity and disappearance of physiological effects *in vivo* are identical for the two inhibitors.

5. The di*iso*propyl- and di*sec*butyl-esters produce an inhibition of cholinesterase that is not reversible *in vitro*. The duration of action *in vivo* is longer than that of the diethylester and very similar to that produced by DFP.

6. In rats none of these esters inhibit brain cholinesterase *in vivo* in doses which produce a marked decrease of enzyme activity in blood and tissues.

7. Compared with their anticholinesterase activity the diethyl-, di*iso*propyl-, and di*sec*butyl-esters are much less toxic than neostigmine, TEPP, DFP, or the dimethyl-ester.

8. The evidence presented provides strong support for the theory that inhibition of cholinesterase by TEPP and DFP is due to a dialkylphosphorylation of the enzyme and that the duration of action of the inhibition depends on the nature of the alkyl groups.

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INHIBITION OF AMINE OXIDASE BY ANTIHISTAMINE COMPOUNDS AND RELATED DRUGS

BY

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Despite the considerable amount of work on the pharmacology of antihistamine drugs since their introduction by Bovet and Staub (1937), relatively little appears to be known concerning the actions of these substances on enzymes. From a consideration of the pharmacological properties of the antihistamines, the enzymes which would be most likely to be affected are histaminase and amine oxidase.

Kapeller-Adler (1949) studied the effect of six antihistamine drugs on a purified hog kidney histaminase preparation, and found that four of them caused no change in enzymic activity, although two did partially inhibit this enzyme. In the course of earlier unpublished experiments on amine oxidase Thompson and Tickner observed that diphenhydramine hydrochloride inhibits this enzyme. Schuler (1950) has also shown that antistin inhibits amine oxidase. Search of the literature showed that potentiation of adrenaline was a common feature of antihistamine drugs. A more systematic study of this inhibitory effect was therefore made. It is the purpose of this paper to show that all antihistamine drugs so far tested are inhibitors of amine oxidase.

Materials and Methods

The following inhibitors were used:

1. Diphenhydramine hydrochloride (Benadryl, Parke Davis & Co.).
2. Tripelennamine hydrochloride (Pyribenzamine, Ciba Laboratory).
3. Antazoline sulphate (Antistin sulphate, Ciba Laboratory).
4. Mepyramine hydrogen maleate (Anthisan, May & Baker Ltd.).
5. Promethazine hydrochloride (Phenergan, May & Baker Ltd.).
6. Phenindamine hydrogen tartrate (Thephorin tartrate, Roche Products Ltd.).
7. Piperidinomethyl-benzodioxane (933F).
8. N : N-dibenzyl-β-chloroethylamine (Dibenamine hydrochloride, Smith, Kline & French).

The source of the amine oxidase preparation was fresh rat liver. In two experiments guinea-pig kidney and rabbit artery were used.

Tyramine hydrochloride (L. Light & Co.) in 0.01 M solution was used as substrate throughout except in those experiments in which the relation between substrate concentration and oxidation ratio was studied.

The method employed was as follows. Albino rats of either sex were stunned and decapitated. The liver was removed, weighed, and homogenized in 0.067 M-phosphate

buffer (*p*H 7.3) so that each ml. of homogenate contained 100 mg. fresh wt. of liver; 2 ml. homogenate (=200 mg. of liver) were placed in the main chamber of a Warburg flask. The inhibitors were dissolved in glass-distilled water and brought to *p*H 7.3 with N-HCl or N-NaOH as required. The final concentrations of inhibitor varied from 2×10^{-5} to 2×10^{-2} M, and the actual volumes of solution added to the main chamber varied between 0.1 and 0.5 ml.; 0.2 ml. of 0.01 M-tyramine hydrochloride was placed in the side bulb. The period of time during which the drug remained in contact with the enzyme preparation before the addition of substrates averaged 15 min. 0.2 ml. KCN solution was added to the main chamber to give a final concentration of 0.001 M; in the centre well was placed 0.2 ml. of a mixture of equal volumes of 2 M-KCN and 0.002 M-KOH.

The level of activity of the uninhibited enzyme was determined in each experiment, controls being included for cyanide-insensitive residual respiration. Where the inhibiting compound was in the form of a salt, the sodium salt of the anion was also added to the control flasks in the same molarity.

The flasks were gassed with O_2, equilibrated at 38° C. for 10 min., the taps closed, and the levels noted. Tyramine was tipped into the main chamber and 30 min. later the levels again read.

When guinea-pig kidney was used as the enzyme source, the experiment was continued for 60 min., and with rabbit arteries 120 min.

In determining the type of inhibition the substrate concentration varied from 10^{-3} to 10^{-2} M.

Results

The results are shown in Table I. It will be seen that all the substances investigated inhibit the enzyme, the degree of inhibition being proportional to the log concentration of the drug. In the higher concentrations there was usually little

TABLE I

Inhibitor	Molar concentration		
	10^{-4}	10^{-3}	10^{-2}
	% inhibition		
Diphenhydramine	22	49	79
Tripelennamine	18	44	82
Antazoline	9	36	77
Mepyramine	9	32	81
Promethazine	25	48	93
Phenindamine	10	45	—
933F	0	22	74
Dibenamine	0	12	—

difference between the drugs, but in the region of 10^{-4} M some quantitative differences did emerge, the most striking of which was the lack of inhibition by 933F and by dibenamine.

Dibenamine, however, because of its very low solubility in aqueous solutions at *p*H 7.3, could be studied only over a very limited range of concentrations, and results with it cannot be as reliable as with the other drugs.

Fig. 1 shows the results obtained for diphenhydramine with three different tissues from three different species. The purpose of these experiments was to show that inhibition of this enzyme is not restricted to one species or one tissue.

The type of inhibition exerted by the antihistamines studied was investigated by plotting the reciprocals of the reaction velocity at various substrate concentrations in presence and absence of inhibitor (Lineweaver and Burke, 1934). Parallel lines were obtained (Fig. 2) showing that the inhibition was non-competitive.

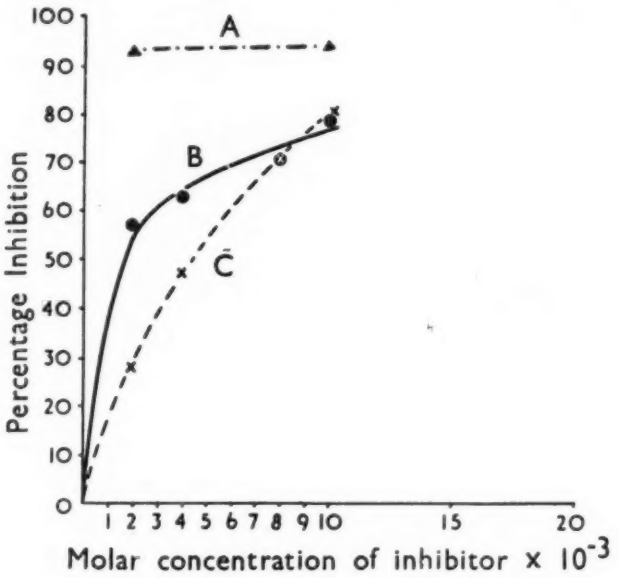

FIG. 1.—Inhibition of amine oxidase from different sources by diphenhydramine. Curve A: minced rabbit aorta; curve B: rat liver homogenate; curve C: guinea-pig kidney homogenate.

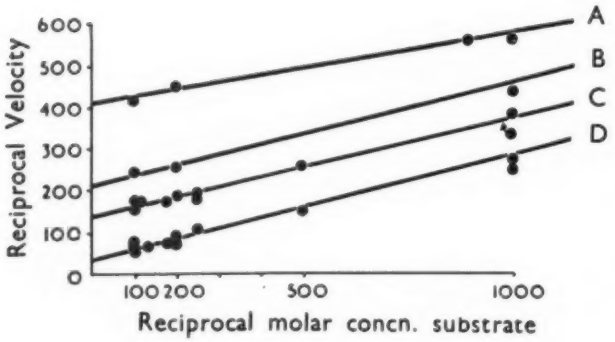

FIG. 2.—Non-competitive nature of inhibition of amine oxidase (rat liver) by diphenhydramine. Ordinates: reciprocal velocities (μl.O_2/g. tissue/hr.). Abscissae: reciprocal molar concentrations of substrate. Concentrations of diphenhydramine hydrochloride: A, 5×10^{-3}M; B, 3×10^{-3}M; C, 10^{-3}M; D, none.

DISCUSSION

The numerous substances which inhibit amine oxidase include sympathomimetic drugs such as amphetamine (Pugh and Quastel, 1937) and ephedrine (Blaschko, Richter, and Schlossman, 1937), local anaesthetics such as cocaine, cinchocaine (Philpot, 1940), and amethocaine (Tickner, unpublished results), and sympathomimetic and sympatholytic imidazolines (Meier, 1950).

Many of these substances imitate or potentiate adrenaline and some antagonize its actions ; these effects have been explained as being due to inhibition of amine oxidase or to combination with adrenaline receptors respectively.

Antihistamine substances have been shown to potentiate adrenaline (e.g., De Cuyper, 1946 ; Parrot, 1943 ; Loew, MacMillan, and Kaiser, 1946 ; Sherrod, Loew, and Schloemer, 1947 ; Sherrod, Schloemer, and Loew, 1946 ; Yonkman, Chess, Mathieson, and Hansen, 1946), or to produce a rise in blood pressure (e.g., Winder and Thomas, 1947 ; Orias, Gilbert, and Brooks, 1949 ; Haley and Harris, 1949 ; Allardyce, Salter, and Rixon, 1951).

The present observations made *in vitro* may explain these side-effects, particularly as the inhibition is non-competitive, unlike that due to ephedrine and the local anaesthetics.

As Tripod (1940) has pointed out the quantitative relationship between the anaesthetic power of local anaesthetics and their sympathomimetic action is not known. Nevertheless it is of interest that the antihistamine drugs studied are both inhibitors of amine oxidase and also local anaesthetics (Brach, 1946 ; Climento, Homburger, and Mosser, 1941 ; Dews and Graham, 1946 ; Halpern, 1942 ; Loew, Kaiser, and Moore, 1945 ; Rosenthal and Minard, 1939 ; Lehmann, 1948).

Another property of these drugs shared with the local anaesthetics is that of having a quinidine-like action on the heart (Dews and Graham, 1946 ; Dutta, 1949). For this reason the effects of quinidine and of atropine were tested on amine oxidase. No inhibition was observed either at 10^{-3} or 10^{-4} M under the conditions described above.

Finally the antihistamines have been used by a number of workers in pharmacological experiments, involving adrenaline, in order to eliminate the effects of histamine. Their sympathomimetic and sympatholytic side-actions indicate that conclusions from such experiments should be drawn with caution.

Summary

1. Six antihistamine drugs and two anti-adrenaline drugs inhibit amine oxidase.

2. The possible relationship between this finding and the pharmacology of these drugs is discussed.

My thanks are due to Professor R. H. S. Thompson for assistance and advice, to Mr. R. Adams for technical assistance, intelligently and assiduously given. I also have to thank Professor J. M. Robson for a gift of 933F, Mr. J. B. Blaikley for a supply of dibenamine, Dr. C. J. Wrigley, of Roche Products Ltd., for a sample of pure thephorin tartrate, and the Central Research Fund of the University of London for a grant for the purchase of some of the manometric equipment.

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SOME ACTIONS OF HEXAMETHONIUM AND CERTAIN HOMOLOGUES

BY

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A great deal of attention is being devoted to the clinical potentialities of the alkamethonium compounds (Paton and Zaimis, 1951 ; Barlow and Ing, 1948), in particular hexamethonium and pentamethonium, on account of their ability to paralyse transmission in autonomic ganglia. Although tetraethylammonium had previously been studied over many years (see review by Moe and Freyburger, 1950), its application in therapeutics has been limited because of its relatively weak action and short duration. Hexamethonium has shown greater possibilities in hypertension, and the work of Kay and Smith (1950) pointed out the possible value of this drug in the treatment of peptic ulcer ; this work prompted some of our initial investigations on gastric motility and secretion. One intriguing aspect was the fact that certain ethyl homologues (Chou and de Elio, 1947) displayed marked differences in ganglionic-blocking potency, which led us to examine the effects of successive replacement in hexamethonium of one or more methyl groups by ethyl groups on each of the two quaternary nitrogen atoms.

METHODS

The methods are described under two headings: (*a*) main actions, and (*b*) gastric motility and secretion.

Main actions.—The effects on sympathetic ganglia were determined on the superior cervical ganglion ; the sustained contractions of the nictitating membrane of the cat under chloralose were recorded, preganglionic stimulation of the cervical sympathetic with maximal square wave stimuli at a frequency of 20 per sec. and a pulse length of 0.1 to 1.0 msec. being used. Effects on parasympathetic ganglia were assessed (1) on the peristaltic reflex of the guinea-pig ileum (Feldberg and Lin, 1949), and (2) by determining the intravenous dose in cats under chloralose required to reduce by about 50 per cent the fall of blood pressure on vagal stimulation, either a faradization current or square wave stimuli being used. Experiments on salivary flow were performed in cats under chloralose (Bülbring and Dawes, 1945). Actions on the cardiovascular system were studied (1) by determining the coronary outflow in the heparinized dog under chloralose with a Morawitz cannula introduced into the coronary sinus, any change in the blood pressure being prevented by a counterbalancing pressure in a reservoir of large volume (50 litres) attached to the femoral artery (Krayer and Verney, 1936) ; (2) on the isolated cat's heart (Langendorff's method), the outflow being recorded by Thorp's impulse counter ; (3) on the vessels of the hind limb of the dog perfused with heparinized blood by a Dale-Schuster pump. Neuromuscular paralysis was examined by means of the phrenic nerve-diaphragm preparation of the rabbit (Wien, 1948) ; by the rabbit head-drop method ; and by the sciatic nerve-gastrocnemius muscle preparation in cats under chloralose (Bülbring and Burn, 1942).

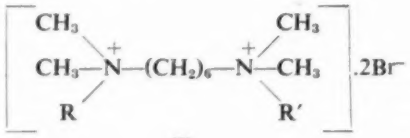
Gastric motility and secretion.—Cats, anaesthetized with chloralose or decerebrate, and rabbits anaesthetized with urethane served to examine the effects of hexamethonium on gastric motility. A rubber balloon filled with water was placed inside the stomach through an incision in the upper end of the duodenum, a levelling bulb was attached, and the pressure within the balloon adjusted to between 8 and 15 cm. of water ; a record of the movements was obtained with a piston volume recorder. Stimulation of the left peripheral vagus with a faradization current afforded a means of observing effects on hypermotility.

Dogs were anaesthetized either with chloralose or with a mixture of chloralose-urethane (1 : 10), and prepared as described by Babkin (1950) for gastric secretion experiments. The duodenum was ligated at the pylorus ; a rubber tube with a non-corrodable metal flange was tied into an opening made in the cardiac end of the stomach on the greater curvature and brought outside the abdominal wall by a stab wound, the fistula being so placed that good drainage resulted. The right vago-sympathetic trunk was stimulated by bursts of induction shocks at a frequency of 20 per second, interrupted 15 times a minute with a Lewis rotary contact. The peripheral end of the nerve trunk was drawn into a fluid electrode consisting of a piece of glass tubing, 2 to 3 mm. bore, into which were inserted two silver plates, and through which passed a slow stream of Ringer's solution ; the nerve could then be stimulated for long periods without fatigue. The blood pressure was recorded from the femoral artery ; the volume of gastric juice was measured every 15 minutes ; free and total acidity were determined by titration using thymol blue as indicator ; and peptic activity was estimated by Hunt's method (1948). In some experiments gastric juice of high acidity but low peptic power was obtained by infusing histamine intravenously at a constant rate of 2.5 μg./kg./min. The compounds examined were prepared by Barber and Gaimster (1951). All injections were intravenous unless otherwise stated.

RESULTS

Hexane-1 : 6-bis-trialkylammonium compounds (homologues of hexamethonium)

Higher homologues of hexamethonium were examined, in which R and R′ in the formula I were ethyl, *n*-propyl, *iso*propyl, *n*-butyl, *iso*butyl, or allyl.



(I)

The results given in Table I show that all the higher homologues were considerably less potent in blocking transmission in autonomic ganglia with the remarkable exception of the bis-ethyldimethylammonium compound which possessed greater activity than hexamethonium on both sympathetic and parasympathetic ganglia (Figs. 1 and 2). We found that reproducible results were easier to obtain with guinea-pig than with rabbit intestine for peristaltic reflex experiments. Even so, the results of an assay might vary during the course of an experiment ; in one experiment the bis-ethyldimethylammonium homologue was initially about twice as potent as hexamethonium, whereas later it was more than four times as potent. An evaluation based on the mean of several experiments showed the homologue to be about twice as potent as hexamethonium.

TABLE I

HOMOLOGUES OF HEXAMETHONIUM

$$R(CH_3)_2\overset{+}{N}—(CH_2)_6—\overset{+}{N}(CH_3)_2R'\}2Br^-$$

Substituent groups R = R′	Relative toxicity (mice) i.v.	Relative potency (paralysing ganglionic transmission)	
		Sympathetic (cat's superior cervical)	Parasympathetic (guinea-pig ileum)
Methyl (hexamethonium)	1.0	100	100
Ethyl	2.3	150	200
n-Propyl	2.9	<5	<5
*iso*Propyl	2.7	15	10
n-Butyl	6.0	<4	<2
*iso*Butyl	2.5	<4	4
Allyl	2.6	4	4

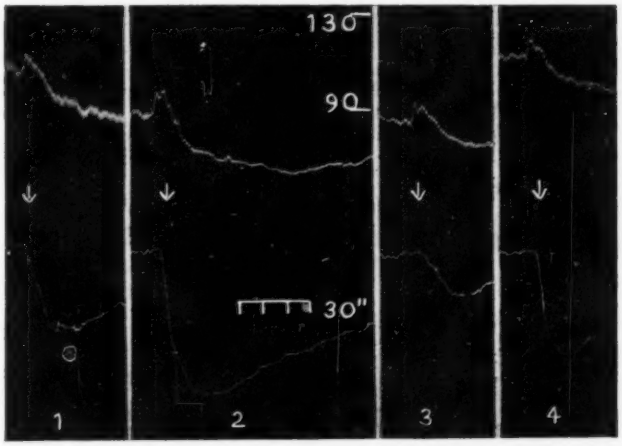


FIG. 1.—Cat, chloralose. Upper record, blood pressure. Lower record, contraction of nictitating membrane on preganglionic stimulation of cervical sympathetic at 20 shocks/sec. Compounds injected intravenously. (1) 0.4 mg., (2) 0.8 mg. bisethyldimethylammonium homologue of hexamethonium bromide, (3) 0.4 mg., (4) 0.8 mg. hexamethonium bromide. The former compound was about 1½ times as potent as hexamethonium.

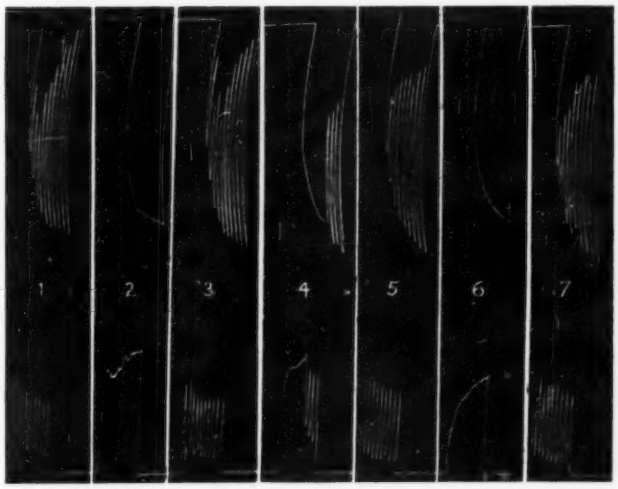


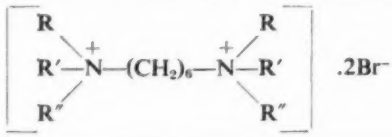
FIG. 2.—Guinea-pig ileum. Circular (upper record) and longitudinal (lower record) movements on stimulation by rise of intra-intestinal pressure of 2.5 cm. of water. (1), (3), (5), and (7) control responses; (2) 50 μg. bisethyldimethylammonium homologue, (4) 200 μg., (6) 300 μg. hexamethonium added to the bath.

The relative toxicity figures are given in terms of the LD50 determined intravenously in mice ; the average lethal doses of hexamethonium dibromide and the bis-ethyldimethylammonium homologue were 0.05 mg./g. and 0.022 mg./g. respectively. The latter compound was more toxic subcutaneously (3.2 times) and orally (1.7 times) as well as intravenously (2.3 times). The cause of death was respiratory failure, and may have been due to a central or neuromuscular-paralysing action rather than to an excessive fall of blood pressure caused by the removal of autonomic vascular control.

The effect of further replacement in hexamethonium of methyl by ethyl substituents was then investigated (Table II).

TABLE II

THE SUCCESSIVE REPLACEMENT OF METHYL BY ETHYL GROUPS IN HEXAMETHONIUM



Substituent groups			Relative toxicity (mice) i.v.	Relative potency (paralysing ganglionic transmission)		
				Sympathetic	Parasympathetic	
R	R′	R″		(Cat's superior cervical)	(Guinea-pig ileum)	(Cat's blood pressure)
Me	Me	Me	1.0	100	100	100
(hexamethonium)						
Et	Me	Me	2.3	150	200	200
Et	Et	Me	4.0	75	100	100
Et	Et	Et	20.0	<5	<50	<50 (paralysis of respiration)

Similar results were obtained by the substitution of methyl by ethyl groups in pentamethonium ; maximum and minimum potencies for paralysis of transmission in autonomic ganglia were found in the bis-ethyldimethylammonium and bis-triethylammonium homologues respectively. The low ganglionic blocking activity of the bis-triethylammonium homologues is in accord with the findings of Chou and de Elio (1947), who, however, used short periods of stimulation instead of sustained stimulation of the cervical sympathetic in their experiments on the perfused ganglion. In addition, we observed that the bis-triethylammonium homologue of hexamethonium had a much greater effect than the parent drug in paralysing neuromuscular transmission (see Figs. 9 and 10). The implication of these findings is discussed later on.

Mode of action

The increased potency of the bis-ethyldimethylammonium homologue was achieved without any significant alteration in its type of action. Thus on the isolated guinea-pig ileum it prevented the contractions produced by nicotine without modify-

ing those due to acetylcholine, histamine, or pilocarpine (Fig. 3), and in the cat post-ganglionic stimulation of the cervical sympathetic was unaffected during a paralysis of pre-ganglionic stimulation (Fig. 4). It may also be mentioned that in the dog (chloralose) the pressor action of nicotine was blocked and that of adrenaline slightly enhanced by both hexamethonium and the homologue.

FIG. 3.

FIG. 4.

FIG. 3.—Guinea-pig ileum. Normal control contractions with (1) 0.4 μg. acetylcholine, (2) 20 μg. pilocarpine, (3) 100 μg. nicotine, (4) 3 μg. histamine; and 1′ to 4′, contractions with same drugs respectively but after the previous addition to the bath on each occasion of 1 mg. bis-ethyldimethylammonium homologue of hexamethonium.

FIG. 4.—Cat, chloralose. Contraction of nictitating membrane. At white dot postganglionic stimulation of cervical sympathetic for 5 sec.; at × sustained preganglionic stimulation. Intravenous injection of 0.5 mg. of bis-ethyldimethylammonium homologue of hexamethonium at arrow paralyses preganglionic stimulation, but leaves postganglionic stimulation unaffected.

Action on salivary secretion

Though hexamethonium has a specific paralysing effect on autonomic ganglionic transmission, differing in its mode of action from atropine, evidence has been found of a very weak inhibitory action on salivary secretion evoked by intravenous infusion of carbamylcholine in the cat. A good effect is shown in Fig. 5, No. 1, where it was several thousand times weaker than atropine; even this response was not consistently obtained, since in some experiments there was very little inhibitory action (Fig. 5, No. 2). Tetraethylammonium had a transient action which was more consistent and about four times greater than that of hexamethonium (Fig. 5, No. 3).

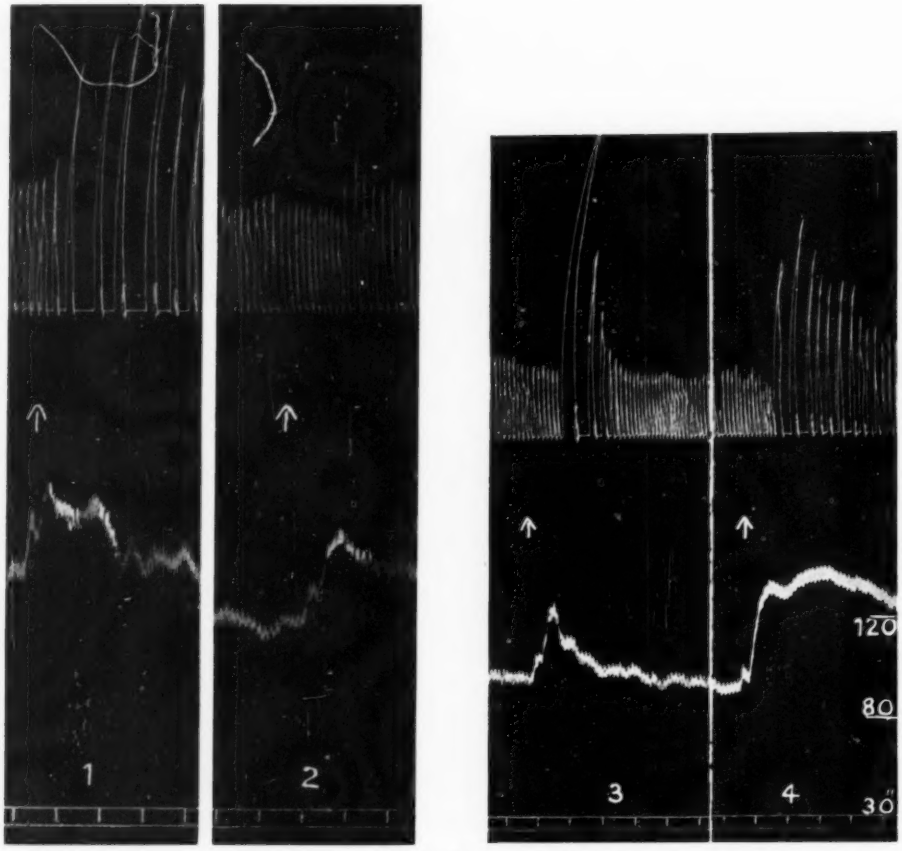


FIG. 5.—Cat, chloralose. Upper record, salivary flow from Wharton's duct on intravenous infusion of carbamylcholine. Lower record, blood pressure. Intravenous injections of (1) 8 mg., (2) 4 mg. hexamethonium bromide, (3) 2 mg. tetraethylammonium bromide, (4) 3 μg. atropine sulphate.

Mydriatic effects

Tetraethylammonium is known to cause mydriasis (Heymans and Hoorens, 1949), and one of the side-effects first noted clinically with hexamethonium was blurring of vision. In view of this we examined the possible mydriatic activity of hexamethonium by the method used by Ing, Dawes, and Wajda (1945), and the results are shown in Table III. Since atropine produced a maximum effect within 20 minutes, and hexamethonium produced its maximum response within 10 minutes, the dose-action curves were plotted with the figures for maximal effects (Fig. 6). Although a linear log dose-response relationship is obtained with atropine, the curve for hexamethonium was non-linear (by analysis of covariance), perhaps on account of the different way in which it exerts its mydriatic action. In another experiment the pupil sizes after 10 minutes were 12.3, 15.3, 18.8, 29.2, and 39.8 with doses of 2.9, 4.4, 6.7, 10.0, and 15.0 μg /g. hexamethonium respectively. A comparison between hexamethonium and tetraethylammonium could not justifiably be made, since the latter compound had a much flatter slope, and with the highest dose, which approximated to the toxic level, the effect was submaximal ; this result might have been due to the stimulant nicotine action of the drug.

TABLE III

THE MYDRIATIC EFFECT OF HEXAMETHONIUM IN MICE

(10 mice on each dose level; compounds injected intraperitoneally)

Compound	Dose μg./g.	Size of pupil (arbitrary divisions of microscope scale)			
		0	10 minutes after injection	20 minutes after injection	30 minutes after injection
Hexamethonium bromide..	5.0	10.7	12.0	10.1	7.0
	10.0	8.3	23.8	18.7	12.2
	20.0	9.0	28.2	28.1	16.8
	40.0	9.1	52.1	44.0	36.5
Atropine sulphate	0.1	10.5	14.4	16.4	17.7
	0.2	8.5	20.8	31.9	28.7
	0.4	8.7	52.2	45.2	43.3
Tetraethylammonium bromide	20.0	12.3	18.1	15.8	10.7
	40.0	11.4	21.1	12.6	10.1

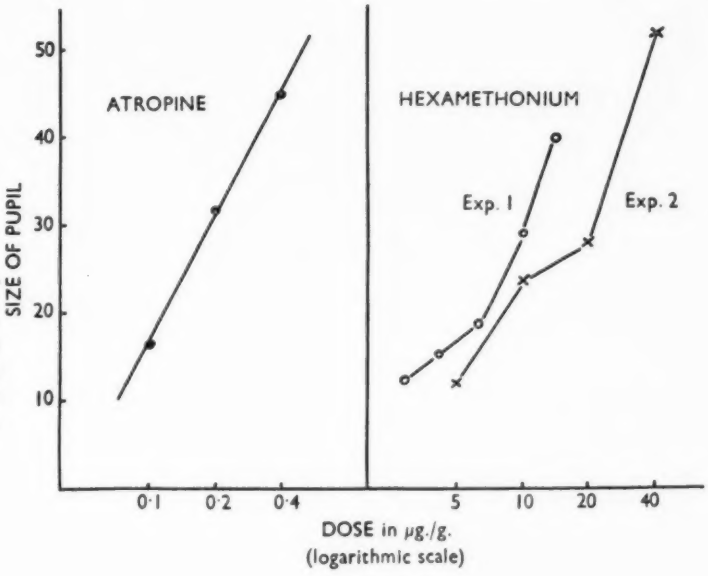


FIG. 6.—Mydriatic action of hexamethonium in mice. Log. dose-response curves for atropine sulphate and hexamethonium bromide (two separate experiments).

Action on the vagus

Hexamethonium reduced or inhibited the fall of blood pressure on stimulation of the right vagus peripherally in the cat, but the fall of blood pressure and slowing of the heart caused by acetylcholine were unaffected. In this way it differed from tetraethylammonium, which did inhibit the bradycardia but not the fall in blood pressure due to acetylcholine.

The bis-ethyldimethylammonium homologue of hexamethonium had an action like that of hexamethonium itself (Fig. 7); it abolished the fall of blood pressure on

vagal stimulation, but had no effect on the bradycardia or fall of pressure due to acetylcholine. The intravenous dose for a 50 per cent reduction in the fall of blood pressure was 1 to 2 mg./kg. for hexamethonium and 0.5 to 1 mg./kg. for the homologue. A vagal block with either compound was demonstrable also in the dog anaesthetized with chloralose-urethane (Fig. 8).

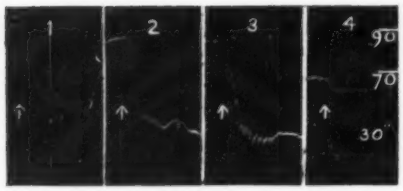


FIG. 7.

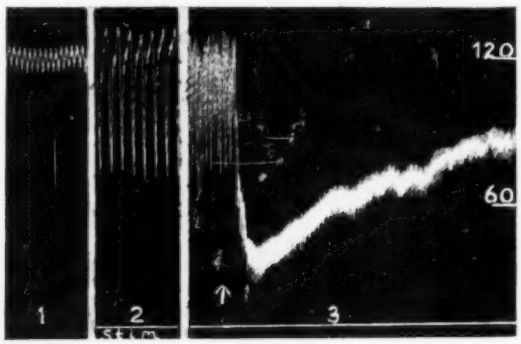


FIG. 8.

FIG. 7.—Cat, chloralose. Record of blood pressure. At (1) and (4) stimulation of peripheral end of right vagus at 20 shocks/sec. for 10 sec. At (2) and (3) intravenous injection of 40 μg. acetylcholine. Between (2) and (3) 0.5 mg./kg. bis-ethyldimethylammonium homologue of hexamethonium.

FIG. 8.—Dog, chloralose-urethane. Record of blood pressure from femoral artery. (1) Before stimulation of vago-sympathetic trunk; (2) and (3) during faradic stimulation of vago-sympathetic trunk, secondary coil at 3 cm.; (3) intravenous injection of 0.5 mg./kg. hexamethonium bromide.

Neuromuscular paralysis

Rabbit phrenic nerve-diaphragm.—The relative potencies for hexamethonium, the bis-ethyldimethyl, bis-diethylmethyl, and bis-triethylammonium compounds were 100:150:200:1600 respectively (Fig. 9). Compared with potent neuromuscular blocking compounds a large dose of hexamethonium is required to produce neuromuscular paralysis, and it is practically free from this action when exerting its main effect—paralysis of autonomic ganglia. The bis-triethylammonium homologue, on the other hand, has appreciable neuromuscular blocking properties.

Rabbit head-drop.—The rabbit head-drop method was used to confirm the results obtained on the isolated nerve-muscle preparation, and the approximate doses required on intravenous injection were 26 mg./kg. for hexamethonium, 21 mg./kg. for the bis-ethyldimethylammonium homologue, 6 mg./kg. for the bis-diethylmethylammonium, and 1 mg./kg. for the bis-triethylammonium compounds. The relative potencies were therefore 100:124:433:2600, which are similar to the values found on the nerve-muscle preparation.

Cat sciatic-gastrocnemius.—These experiments provided additional evidence of the neuromuscular-blocking action of the bis-triethylammonium compound in contrast to the lack of such an effect with either hexamethonium or the bis-ethyldimethylammonium homologue (Fig. 10).

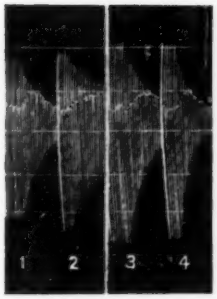


FIG. 9.

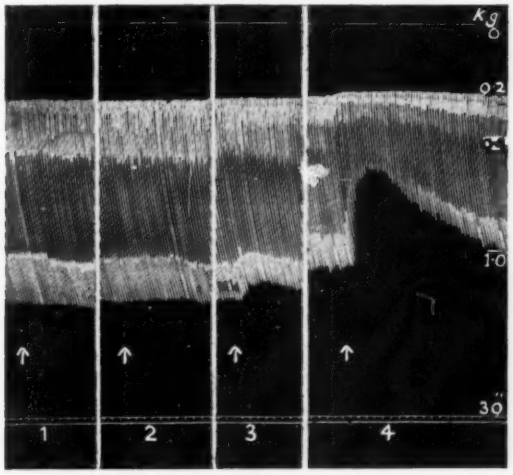


FIG. 10.

FIG. 9.—Rabbit phrenic nerve-diaphragm preparation at 37° C. Record of muscle contractions on maximal stimulation of nerve with 6 shocks/min. (1) and (3) 50 mg. hexamethonium bromide, (2) and (4) 2 and 4 mg. bis-triethylammonium homologue. The bis-triethylammonium homologue was 16 times as potent as hexamethonium in producing neuromuscular block.

FIG. 10.—Cat, chloralose. Record of twitch tensions of gastrocnemius on supramaximal stimulation of sciatic nerve with square wave stimuli 6/min. and 1 m.sec. duration. Intravenous injection of (1) 25 mg. hexamethonium bromide, (2) 25 mg. bis-ethyldimethylammonium homologue, (3) 5 mg. and (4) 10 mg. bis-triethylammonium homologue.

Action on the heart

Isolated cat's heart.—The cat's heart was used, perfused with oxygenated Locke's solution at 37° C., in which the effect of adrenaline was normally dilator on the coronary vessels. Hexamethonium iodide, in a dose of 1 mg. injected into the side-arm leading to the perfused aorta, had no effect on the beat or outflow (Fig. 11, No. 1). Only when the amount was increased to 20 or 40 mg. was an effect produced,

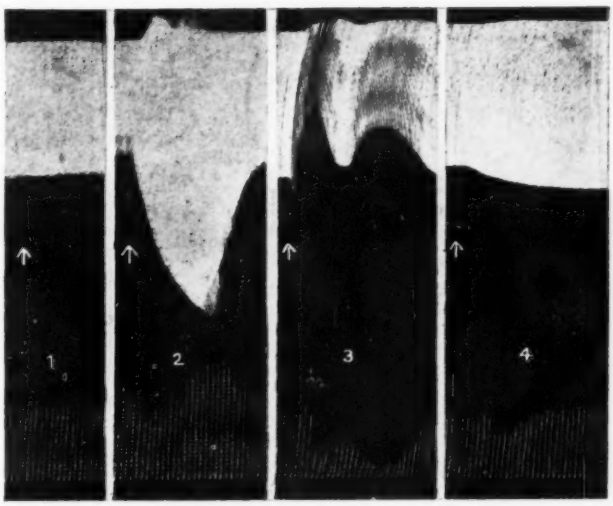


FIG. 11.—Isolated cat's heart, perfused with Locke's solution at 37° C. Upper record, movements of heart; lower record, coronary outflow—an increase in outflow upwards. (1) 1 mg. and (4) 20 mg. hexamethonium iodide, (2) 0.2 μg. adrenaline, (3) 4 μg. acetylcholine.

resulting in an increase in the amplitude and a transient constriction, followed by a dilatation, of the coronary outflow (Fig. 11, No. 4); these concentrations are far in excess of those ever likely to be attained in practice.

Coronary outflow in dog.—Changes in the coronary outflow were observed in the dog (chloralose) by the introduction of a Morawitz cannula, the arterial pressure being maintained constant; the volume of blood flowing from the sinus was measured as required and returned to the animal by the femoral vein. The compounds were given intravenously, and the results are shown in Table IV: the figures

TABLE IV

The effect of hexamethonium bromide (A) and of the bis-ethyldimethylammonium homologue (B) on the coronary flow in dogs

Exp.	Dog kg.	Arterial pressure mm. Hg	Drug	Dose mg./kg.	Coronary outflow* (ml./min.)			Remarks
					Initial	3 min.	10 min.	
1	14	110	A	0.25	76	124	88	Adrenaline, 30 μg., increased the outflow from 92 to 144; hexamethonium (A) increased the heart rate from 148 to 176 a minute
			B	0.25	96	92	92	
2	11	60	A	1.00	54	44	56	
3	16	120	A	0.25	92	90	96	Hexamethonium (A) increased the heart rate from 160 to 180 a minute
			A	1.00	100	93	94	
			B	0.50	78	72	81	
4	12	90	A	1.00	92	78	90	
			B	1.00	80	74	84	
5	11	120	A	1.00	120	140	132	Adrenaline, 25 μg., increased the outflow from 88 to 102
			B	1.00	114	132	126	

* Coronary flow represents 60 per cent of actual output; figures are means of several observations.

are the means of several observations. It was, however, difficult to obtain a clear picture of the effect of hexamethonium on the coronary outflow, since in some dogs there was no change, while in others there was a slight decrease or increase; Wégria (1951) similarly found in several experiments with tetraethylammonium both a decrease and an increase in the coronary flow. The results were further complicated by the fact that once an effect was obtained it was not always possible to repeat it later in the experiment, though adrenaline repeatedly increased the outflow. At least 30 minutes elapsed between each dose in order to allow the effects of each compound to wear off and the outflow to return to normal. On the whole the effects were relatively small and transitory, and no marked constriction of the coronary outflow was ever observed; in some instances hexamethonium might even have a beneficial action by increasing the coronary outflow.

Actions on the vessels

Even though the depressor action of hexamethonium appeared to be mainly accounted for by the blocking of impulses passing through the autonomic ganglia

and consequent removal of sympathetic tone, evidence was required of any possible direct effect on the peripheral vessel walls. To obtain this evidence the vessels of one hind limb of the dog were perfused by a Dale-Schuster pump with heparinized blood, and observations were made on the peripheral resistance and venous outflow. Fig. 12 shows a result, obtained on several occasions, of an experiment where a slight vasodilatation was produced on the injection of hexamethonium into the arterial supply to the limb. The dilatation did not depend on the tone of the vessel walls, since no greater response was obtained when the peripheral resistance was raised by the addition of adrenaline to the reservoir of blood in the system. From these results it was concluded that the vasodilator effects were small and could play no great part in the production of hypotension by hexamethonium.

Gastric motility

In the cat, either decerebrate or under chloralose, hexamethonium given intravenously caused a marked contraction of the stomach. It prevented the cardiac but not the gastric effects of vagal stimulation ; the vagal effects on the stomach were even increased and prolonged (Fig. 13). Increasing the pressure inside the balloon prevented the stimulant action of hexamethonium, but the vagal effects on the stomach were prolonged as before (Fig. 14). Atropine had a purely inhibitory effect. Evidence of an inhibitory action of hexamethonium was, however, obtained in the rabbit, in which the spontaneous movements were reduced (Fig. 15). The different results in the cat and rabbit may depend on differences in the functional autonomic innervation of the stomach in the two species ; Brown, McSwiney, and Wadge (1930) showed that the body of the stomach in cats and dogs receives motor and inhibitor fibres from the sympathetic, contraction or relaxation being produced according to the type of stimulation.

To ascertain whether the stimulant action of hexamethonium in the cat was exerted directly on the stomach, observations were made after nicotine, given in sufficient amount to paralyse sympathetic stimuli completely, since the stimulant action might have been due to the preferential blocking of the sympathetic rather than the parasympathetic system. If this assumption were correct it would result in the removal of any sympathetic inhibitory control of the stomach, allowing any excitatory parasympathetic control to manifest itself. An experimental result in support of this contention is shown in Fig. 16, where the intravenous injection of

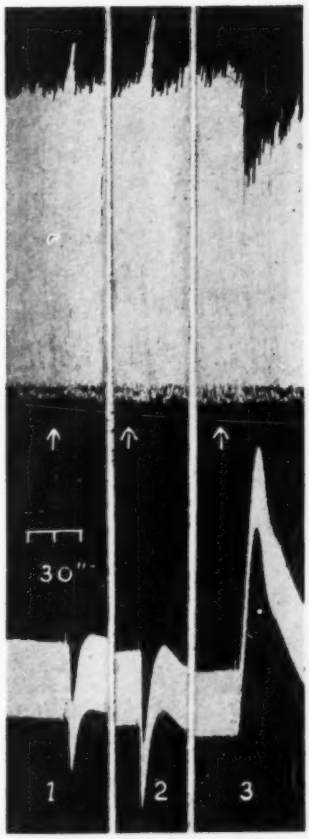


FIG. 12.—Perfusion of vessels of left hind limb of dog with heparinized blood. Upper record, venous outflow; lower record, arterial pressure. Intra-arterial injections of (1) 0.2 mg. hexamethonium bromide, (2) 2 μg. acetylcholine, (3) 1 μg. adrenaline.

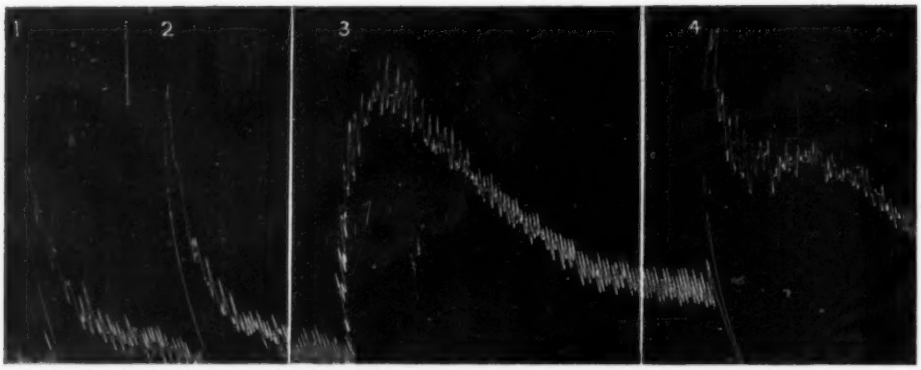


FIG. 13.—Cat, chloralose. Gastric motility, " pressure " in balloon 8–10 cm. water. At (1), (2), and (4) stimulation of left peripheral vagus; at (3) 1.0 mg. hexamethonium bromide intravenously.

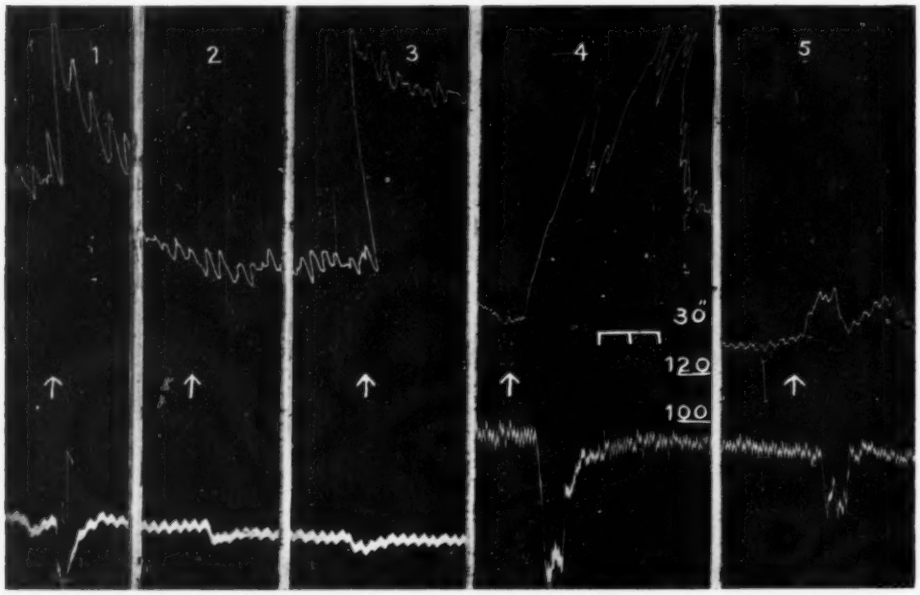


FIG. 14.—Cat, chloralose. Upper record, gastric motility, " pressure " in balloon 15 cm. water; lower record, blood pressure. At (1), (3), (4), and (5) stimulation of left peripheral vagus; at (2) 5.0 mg. hexamethonium bromide. Between (4) and (5) 50 μg. atropine sulphate intravenously.

a dose of hexamethonium which had previously stimulated the stomach movements no longer had this effect after nicotine.

Gastric secretion

On sustained stimulation of the peripheral end of the vago-sympathetic trunk in anaesthetized dogs with bursts of induction shocks every four seconds, a copious flow of gastric juice was produced, the amounts varying from 30 to 180 ml. per hr. A stronger stimulus was required to initiate the flow of gastric juice than that required to slow the heart. The rate of flow varied considerably in different dogs,

but the general trend was the same in all of them. The juice secreted was of high acidity and high peptic power ; after one to two hours the volume and composition remained fairly constant, when it was possible to determine the effect of the intravenous injection of a drug. The cardiac effects of vagal stimulation were inhibited by hexamethonium bromide concurrently with the inhibition of gastric secretion (see Fig. 8), but the effect on the blood pressure usually passed off before the gastric secretion had returned to its original level. There was a graded relationship between the dose of hexamethonium and the extent and duration of the response obtained (Fig. 17) ; the threshold dose for a partial inhibition was 0.06 mg./kg.,

FIG. 15.—Rabbit, urethane. Upper record, gastric motility; lower record, blood pressure. At arrow, 5.0 mg. hexamethonium bromide intravenously.

FIG. 16.—Cat, chloralose. Record of gastric motility, " pressure " inside balloon 8–10 cm. water. (1) and (2) 0.5 mg./kg. hexamethonium bromide intravenously; between (1) and (2) 12 mg. nicotine.

FIG. 17.—Effect of intravenous hexamethonium bromide on the free acid in gastric juice evoked by stimulation of vago-sympathetic trunk in dogs. Maximal response 30–60 min. after injection.

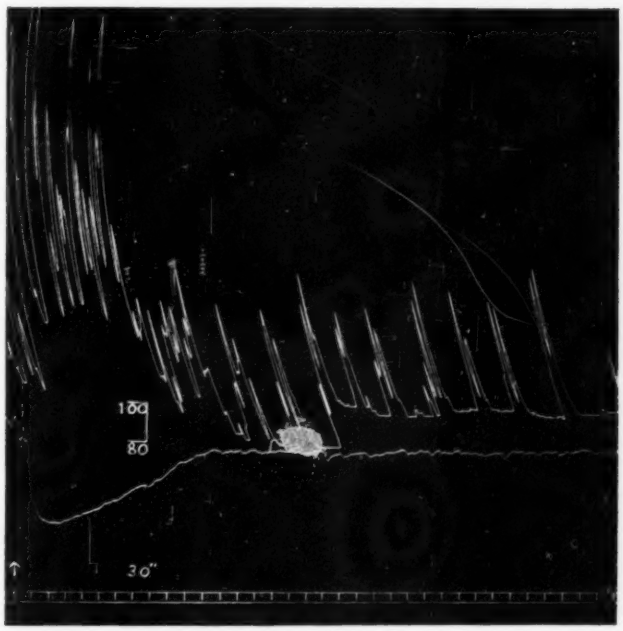


FIG. 15.

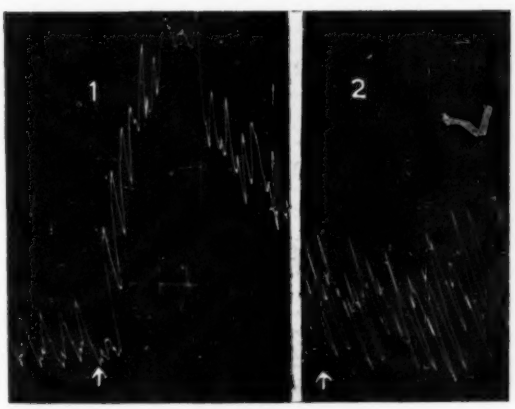


FIG. 16.

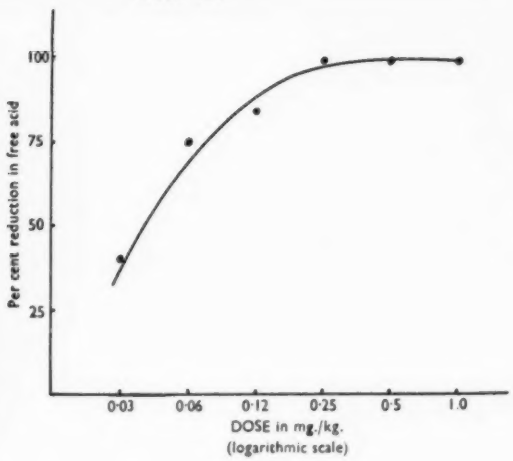


FIG. 17.

while 1 to 2 mg./kg. produced an achlorhydria lasting one or more hours (see Table V). The maximum effect usually occurred within 30 to 60 minutes and the decreases in acidity and in volume closely paralleled one another, but the peptic activity was reduced for a longer period; even three hours after 1 mg./kg. of hexamethonium bromide the peptic power was still only about one half the original figure, although by that time the volume and acidity were restored to their initial values. The bis-ethyldimethylammonium homologue in a comparable dose had a greater effect than hexamethonium itself. In order to produce a comparable effect

TABLE V

GASTRIC SECRETION IN DOGS, EVOKED BY VAGAL STIMULATION

Vol. = Volume of juice in ml. Free A. = Free acid, ml. 0.1N-HCl/100 ml. juice. Total A. = Total acid, ml. 0.1N-HCl/100 ml. juice. Pepsin = Pepsin units (Hunt, 1948). At zero time the mean figures are given for the steady pre-injection level, obtained over several hours

Dog kg.	Dose mg./kg. i.v.		Gastric secretion values							
			0	15	30	45	60	75	90	120
			minutes after injection							
			Hexamethonium bromide							
13	0.03	Vol.	24	15	18	26	21	39		
		Free A.	8	8	5	8	7	8		
		Total A.	140	152	142	156	140	146		
	0.06	Vol.	13	8	9	12	15	17	18	
		Free A.	8	8	7	5	2	7	8	
		Total A.	127	144	130	128	126	140	140	
10	0.12	Vol.	45	10	2	18	28	30	32	
		Free A.	25	20	4	12	18	22	22	
		Total A.	180	184	108	130	155	170	175	
		Pepsin	176		69	114		82	86	
10	0.25	Vol.	8	5	1	0	2	10	8	
		Free A.	8	9	Trace		Trace	6	8	
		Total A.	150	140	74		82	152	166	
10	0.50	Vol.	23	8	2	2	7	8	9	15
		Free A.	17	16	Trace	Trace	5	10	13	16
		Total A.	213	190	72	20	100	120	140	297
		Pepsin	247			95	136		174	
10	1.00	Vol.	10	5	2	1	1	0	0	3
		Free A.	16	16	Trace	Trace	Trace			Trace
		Total A.	140	112	54	30	22			68
		Pepsin	189	102	82					92
			Bis-ethyldimethylammonium homologue							
14	0.06	Vol.	8	9	3	1	4	17	20	
		Free A.	6	5	1	Trace	1	3	6	
		Total A.	150	132	116	130	130	128	156	
			Tetraethylammonium bromide							
14	20.00	Vol.	10	3	1	0	0	1	5	18
		Free A.	38	24	4			Trace	21	34
		Total A.	190	186	98			103	199	187

with tetraethylammonium about 10 to 20 times the dose had to be given. Neither hexamethonium nor the homologue had any influence whatsoever on the secretion evoked by an infusion of histamine ; this secretion was of high acidity but of low peptic power, in contrast to the secretion produced by nervous stimulation.

Excretion in urine

We have not made an extensive study of the absorption and excretion of hexamethonium, but in view of the empirical use of the drug by mouth in clinical practice it was of importance to know whether it is well absorbed by this route. Zaimis's method (1950) was employed, which is similar to that described by Rennick *et al.* (1947) for the estimation of the tetraethylammonium ion. It is based on the production of a coloured acetone-soluble precipitate when ammonium reineckate is added to a solution of a soluble salt of hexamethonium. The test is not specific and can be used for choline and other quaternary ions, but these compounds are normally absent from urine. The method is not very sensitive, and when small amounts have to be determined a biological test, for example the nictitating membrane of the cat, is preferable ; but a still more sensitive and readily applicable method is desirable in order to investigate more fully the metabolism and distribution of the drug in the body.

Rabbits were used, and they were deprived of food the night before an experiment. Hexamethonium iodide was given in solution by stomach tube or intravenously into the marginal ear vein. When given intravenously up to 84 per cent of the dose administered was excreted in the urine within 24 hours. Intramuscular injection also produced good elimination, but much less was recoverable in the urine after oral administration (Table VI).

TABLE VI

URINARY EXCRETION OF HEXAMETHONIUM IN RABBITS

Route	Dose mg./kg.	Per cent excreted (total) in urine during			
		1	2	3	4 days
Intravenous	10	76.5			
Intravenous	50	84.0			
Intramuscular	50	58.0			
Intramuscular	100	78.4			
Oral	250	2.8			
Oral	500	10.3	11.5	13.8	15.4
Oral	500	23.5	25.6	26.8	27.9
Oral	500	9.8	12.1	14.4	15.9
Oral	1,000	4.2	24.8	28.8	—

We have also had the opportunity, through the courtesy of Dr. S. Locket, at Oldchurch Hospital, of examining the urinary excretion of some patients receiving the bis-ethyldimethylammonium homologue of hexamethonium. Eleven patients received oral doses varying from 500 to 3,500 mg. daily, and the urinary excretions within 24 hours varied from 0.2 to 33.8 per cent of the dose administered. In two patients who received parenteral as well as oral treatment it was possible to

compare the merits of the different routes of administration. The first patient excreted 93 per cent of an intramuscular dose of 120 mg., but only 1 to 6 per cent after oral treatment with doses of 500 to 3,500 mg. daily. The second patient received 1,190 mg. subcutaneously over a period of 14 days, and during that time excreted 1,146 mg. in the urine (96 per cent); only 4 per cent was eliminated after 1,000 mg. by mouth, but 33 per cent of an oral dose of 2,000 mg. (which produced a marked reaction) was excreted within three days. Sufficient evidence was not available to make a significant assessment, but it appeared that there was considerable variation from patient to patient, and that in general the major portion of the drug given by either the intramuscular or the subcutaneous routes could be recovered in the urine, whereas a variable and smaller amount was detected after oral treatment. These results are in agreement with those of Milne and Oleesky (1951).

Discussion

Hexamethonium inhibits the transmission of nervous impulses in the autonomic system. The paralysing effect occurs at the ganglion synapse, since postganglionic stimulation remains effective; its action does not extend to the effector organs. Parasympathetic as well as sympathetic ganglionic transmission is affected, though not necessarily to the same extent at all ganglionic junctions. That hexamethonium affects both parts of the autonomic system might be anticipated in view of the close physiological relationship and interdependence of the two parts, but further information is required on the relative effects on different ganglia throughout the body. Evidence is available that the superior cervical ganglion and the ganglia in the stomach may be affected without there being any concurrent action on the blood pressure.

Having noted the remarkable difference in potency between hexamethonium and the bis-triethylammonium homologue, we examined the effect of successive replacement of methyl by ethyl groups. Optimum activity was found in the bis-ethyldimethylammonium homologue, which was one and a half times to twice as potent as hexamethonium in paralysing transmission in both sympathetic and parasympathetic ganglia. This was achieved without any significant alteration in its type of action. The bis-triethylammonium homologue, by contrast, had a neuromuscular paralysing action which was absent in the bis-trimethyl or bis-ethyldimethylammonium compounds.

Of particular interest in the methonium series was the suggestion that maximum activity depended on an optimum chain length and a "fit" between the groupings at each end of the molecule and the receptor groups. It appears to us now that in hexamethonium not only the distance between the two quaternary nitrogen atoms, or inter-receptor distance, but also the *nature* of the terminal groupings is an important factor in determining optimal activity; the relatively simple substitution of ethyl for methyl groups on the two nitrogen atoms exerted a profound influence. The best fit on the receptors might depend on a certain "effective size" which is optimal when the terminal groupings are bis-ethyldimethyl rather than trimethylammonium. This suggestion might be interpreted on the assumption that the groupings associated with the two nitrogen atoms are able to fit on to the receptors when the "effective size" is that found in the trimethyl or ethyldimethyl groups, but that the triethyl grouping

is too large for a good attachment and consequently activity is lost. But it is curious that corresponding with this diminution of potency in blocking autonomic ganglia there is an increase in neuromuscular-blocking activity ; the problem obviously still requires a more complete explanation.

There are some actions of hexamethonium to which we should like to draw attention. It was found that the drug had a very weak and inconsistent inhibitory effect on salivary secretion induced with carbamylcholine, but since this was produced only with relatively large doses it is not necessarily related to the clinical finding of dry mouth ; but we have not investigated the possibility of an inhibitory effect on transmission through the chorda tympani. Of more significance was the relatively potent mydriatic action, presumably caused by paralysis of the ciliary ganglion: this mydriatic action of hexamethonium might well serve as a method of estimation of the drug in body fluids. No marked deleterious action was observed on the heart ; in some experiments there was even an improvement in the coronary circulation. In dogs, where there is a comparatively high degree of vagal tone, vagal block with hexamethonium was sometimes accompanied by a tachycardia which, however, soon passed off. The dilator action on the vessels was slight: it would hardly account for the large fall in blood pressure caused by hexamethonium which must be mainly due to the removal of sympathetic tone.

The effects of hexamethonium on the motility of the stomach were variable, dependent most probably on the composition of the innervation of the stomach in different species. Ambache (1951) has shown that there are two kinds of functionally distinct ganglion cells in the myenteric plexus ; stimulation of the one causes contraction, of the other inhibition, of the intestine. A similar state of affairs may exist in the stomach, and the results of Paton and Zaimis (1951), who found that hexamethonium caused vigorous contractions of the cat ileum, are in accord with our findings on the stomach. In the rabbit the effect was purely inhibitory, resulting in a decrease in tone and movements, but in the cat the main effect was stimulation. This was not a direct action on the stomach, since it was abolished after a full paralysing dose of nicotine. It was most likely due to an interference of gastric nervous control, by the preferential blocking of sympathetic rather than vagal ganglia, which would result in the removal of sympathetic inhibition of the stomach, allowing any vagal stimulant effect to manifest itself.

Although the state of activity of the sympathetic nervous system influences the motility of the stomach, it is problematical to what extent, if any, the sympathetic exerts control over the secretion of gastric juice. But there is no doubt whatsoever that excitability of the vagus influences secretion, and it was found that both hexamethonium and its bis-ethyldimethylammonium homologue inhibited the formation of acid in the gastric juice of the anaesthetized dog, evoked by vagal stimulation ; the volume of juice and its peptic power were likewise decreased. These compounds were without effect, however, on the acid secretion induced by histamine. This is perhaps no more than might be expected, since the inhibitory action is exerted only indirectly on the mechanism of nervous control, not directly on the acid-secreting cells.

Summary

1. A study has been made of certain actions of hexamethonium and of homologues with ethyl substituents, in which one or more methyl groups on each nitrogen atom were replaced by ethyl groups.

2. The bis-ethyldimethylammonium homologue (hexane-1 : 6-bis-ethyldimethylammonium dibromide dihydrate) was one and a half times to twice as potent as hexamethonium in paralysing autonomic ganglionic transmission in both sympathetic and parasympathetic ganglia, and it had a similar type of action. The bis-triethylammonium homologue was much less potent and possessed neuromuscular-blocking properties absent in the other compounds. The bis-diethylmethylammonium compound was equally as active as hexamethonium on parasympathetic ganglia, but slightly less potent on sympathetic ganglia. These structure-action relationships are discussed, and attention is drawn to the importance of the nature of the terminal groupings as well as the distance between the two quaternary nitrogen atoms as a factor determining optimal activity.

3. Certain actions of hexamethonium, such as inhibition of salivary secretion and vasodilatation, were shown to be very slight, and consequently play little part in the main action of the drug. But hexamethonium has an appreciable mydriatic effect, which is most likely due to paralysis of the ciliary ganglion ; a maximal response was attained more quickly than with atropine, which dilates the pupil by a different—peripheral, not ganglionic—action. Hexamethonium had little effect on the heart ; in some instances it improved the coronary circulation and produced a tachycardia, the latter effect caused by the removal of vagal tone.

4. Gastric motility may be excited or inhibited by hexamethonium dependent on the functional innervation of the stomach in different animals. In the cat stomach movements were increased, owing to the removal of sympathetic inhibitory control, without any changes in blood pressure.

5. Gastric secretion in the dog, evoked by vagal stimulation, was inhibited by both hexamethonium and its bis-ethyldimethylammonium homologue. There were reductions in volume, in free and total acidity, and in peptic power ; the extent and duration of these effects were dependent on the dose. No inhibitory effect was exerted on gastric secretion induced with histamine.

6. Considerably less of these compounds was excreted in the urine after oral than after intravenous administration.

It is a pleasure to acknowledge the interest taken in this investigation by Dr. A. J. Ewins and certain helpful discussions with Professor J. H. Burn.

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DEPENDENCE OF THE TOXICITY OF ANTRYCIDE METHYLSULPHATE IN MICE ON THE VOLUME OF A DOSE INJECTED SUBCUTANEOUSLY

BY

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When a drug is injected subcutaneously, its concentration in the blood stream depends on the rate of its absorption. If, therefore, the drug is one that causes immediate toxic effects, their severity will also depend on its rate of absorption. The trypanocidal drug "Antrycide" provides an excellent example. When the soluble methylsulphate is injected subcutaneously in mice, absorption is rapid and most mice will die within a few hours after a dose of 0.8 mg. per 20 g. body weight. When, however, the very insoluble chloride is given, absorption from subcutaneous tissues is so slow that mice will tolerate a dose 150 times as great (Curd and Davey, 1950). Using only the soluble methylsulphate, we have found that its toxicity (as determined by percentage mortality) when injected subcutaneously in mice is significantly influenced by the volume of fluid in which it is given. We believe this to be due to different rates of absorption when different volumes are injected, and therefore to afford another illustration of the dependence of the toxicity of a rapidly acting compound on its rate of absorption.

The subcutaneous dose used, per 20 g. body weight, was 0.75 mg. dissolved in 0.1, 0.2, 0.4, and 0.8 ml. distilled water. The mice were young adults of either sex, ranging in weight from 18 to 26 g. The Table and graph (Fig. 1) below show the results of two similar experiments (on different days), each involving 120 mice, obtained from a different source for each experiment. In each experiment the mice were divided into four equal groups for subcutaneous injection with the volumes of 0.1, 0.2, 0.4, and 0.8 ml. respectively. Care was taken to ensure that each group was treated under the same conditions (except for the difference in dose-volume). The mice had not been fed since the previous day, and, as far as possible, they were all injected at the same time, 10 with one dose-volume, then 10 with the next, and so on, repeating this cycle until all 30 in each group had been treated in each experiment. Signs of toxicity appeared within a few minutes of the injection and consisted of accelerated breathing and general activity and excitability, sometimes followed by a more passive condition than normal but without any obvious paralysis. About three-quarters of the ensuing deaths occurred within an hour of the injection, and were preceded by the sudden onset of violent convulsions a minute or two before the end. No death occurred more than five hours after the injection, and the next day all the survivors appeared to be perfectly normal.

TABLE I

NUMBER OF MICE KILLED, IN RELATION TO NUMBER TREATED, BY SUBCUTANEOUS INJECTION OF 0.75 MG./20 G. ANTRYCIDE METHYLSULPHATE IN VARYING VOLUMES OF AQUEOUS SOLUTION

Volume, in ml.	Exp. 1	Exp. 2	Totals
0.1	$\frac{16}{30}$ (53%)	$\frac{16}{30}$ (53%)	$\frac{32}{60}$ (53%)
0.2	$\frac{10}{30}$ (33%)	$\frac{20}{30}$ (67%)	$\frac{30}{60}$ (50%)
0.4	$\frac{9}{30}$ (30%)	$\frac{13}{30}$ (43%)	$\frac{22}{60}$ (37%)
0.8	$\frac{6}{30}$ (20%)	$\frac{12}{30}$ (40%)	$\frac{18}{60}$ (30%)

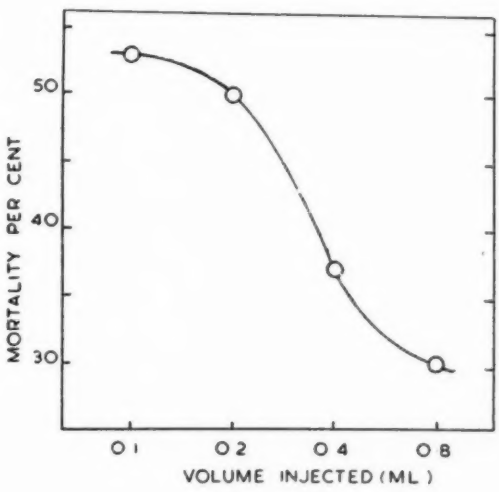

FIG. 1

It will be seen from the Table, which shows the numbers and percentages of deaths, that the mice of Exp. 2 were, on the whole, more affected than those of Exp. 1. This illustrates the well-known variability of results to be expected in toxicity tests with mice obtained from different sources of supply (and treated at different times). There was, however, in each experiment, the same trend from higher mortality in the groups injected with the smaller volumes to lower mortality in those treated with the larger volumes. When the results of the two experiments are combined, as in the last column of the Table and on the graph, the mortalities range from 53 per cent for mice treated with 0.1 ml. to 30 per cent for those treated with 0.8 ml. Applying the probit transformation to the percentages in the last column of the table, the regression coefficient (b) of probits on log volume injected is found to be -0.707, with a standard error of 0.246. The ratio of b

to its standard error is 2.87, and b is therefore significantly different from zero (probability less than 0.01). There is therefore no doubt that our figures show a true relationship between the volume in which the dose was injected and the toxic effect produced.

One possible explanation of the relationship between toxicity and volume injected is that 0.8 ml. is a relatively large amount to introduce into a mouse, whose total blood volume may possibly not exceed 2 or 3 ml. The concentration in the blood that would result from the injection of a compound in such a large volume might therefore be significantly less than would result from an injection of the same dose in a smaller volume. To test this we have treated two further groups, each of 30 mice, from the same source of supply and at the same time, in the following manner. One group received 0.8 ml. saline subcutaneously, immediately followed by another subcutaneous injection, in a different part of the back, of the standard dose of antrycide methylsulphate (0.75 mg./20 g.) in 0.1 ml. water. The other, control, group received only the standard dose in 0.1 ml. water. The percentage mortalities were 57 per cent in the first group and 50 per cent in the controls, and we may therefore exclude the possibility that the lower mortality associated with the larger volumes in the main experiments is to be explained merely by the introduction of excess fluid. It is necessary for this excess to be actually a part of the solvent in which the dose of drug is administered. That delayed absorption probably accounted for the lower mortalities under these circumstances is borne out by the fact that when the compound was injected in 0.8 ml. a swelling was still easily visible at the site of injection about four hours later, while there was no obvious swelling at any time after an injection in 0.1 ml. Apart from mechanical factors, the steeper concentration-gradient between the injected mass and the surrounding tissue-fluids when the compound is given in smaller volumes no doubt increases the rate of absorption, and vital centres are accordingly subjected to the impact of higher concentrations of the drug. Another factor which might help to delay absorption when the larger volumes are used has been suggested to us by Dr. H. R. Ing. This is that the longer period during which the methylsulphate remains under the skin, when it is given in larger volumes, provides a greater opportunity for the interaction with chloride ions of the body fluids to which reference has been made by Curd and Davey (1950). There may consequently be a greater tendency to the formation and local deposition of the sparingly soluble and slowly absorbed chloride salt of antrycide.

Conclusions

Two lessons are to be drawn from these observations, one of general and the other of specific applicability. The outcome of general significance is that when quoting the subcutaneous LD50 of a substance whose lethal action is exercised quickly, it is evidently especially important to state the volume in which the dose was administered. The outcome of specific interest relates to the use of antrycide methylsulphate in veterinary practice. The customary route of administration in cattle is by subcutaneous injection, and when deaths occur they usually do so within 12 hours of the injection. In the light of the present work it might be advisable, when giving a big dose of this salt of antrycide, to inject it in as large a volume as convenient. Admittedly the peak of the resultant curve relating blood concentration to time will tend to be lower than if the dose were given in a smaller

volume, and one cannot yet be sure that this will not slightly reduce the therapeutic effect. It is significant, though, that after subcutaneous injection in the mouse, rabbit, and calf the peak has in fact been shown to be very sharp and to occur within an hour of the injection (Spinks, 1950). Undoubtedly the animal's survival depends on the height of this sharp peak. Trypanosomes, however, are destroyed by exposure to considerably lower blood concentrations than those that kill the host. For example, Spinks quotes that equal efficacy has been reported in the treatment of infected herds in the Sudan by two preparations of antrycide which would give peak plasma concentrations of 30 and 300 μg./litre respectively. It is therefore likely that when a big dose of antrycide methylsulphate is injected the practice of giving it in the largest convenient volume will increase the safety factor without significantly impairing therapeutic efficacy.

We are obliged to Dr. D. J. Finney, Lecturer in the Design and Analysis of Scientific Experiment in the University of Oxford, for advice on the statistical treatment of our results. The work was supported by a grant from the Medical Research Council to E. M. L.

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PHARMACOLOGY OF BENZILIC ACID DERIVATIVES

BY

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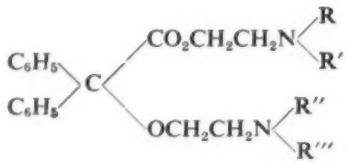
In this paper the results are presented of a pharmacological study of a new series of benzilic esters (" Ro- series ") of type I related in structure both to benadryl (II), one of the early antihistamine drugs (Loew, Kaiser, and Moore, 1945), and to trasentin (III), an established general spasmolytic drug (Meier, 1936 ; Johnson and Reynolds, 1937 ; Goodman and Gillman, 1941). They do not contain the *cyclo*hexane ring present in trasentin-6H. Table I shows the 11 members of the series. The synthesis of these compounds has been described elsewhere (Morrison, Königstein, and Cohen, 1950).



(I)

(II)

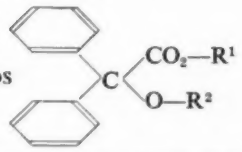
(III)

METHODS

Isolated intestine.—Isolated guinea-pig ileum, suspended in a 2.5-ml. bath (Barsoum and Gaddum, 1935), was used in screening for antihistamine and other spasmolytic activity. A Wellcome " Agla " all-glass micro-syringe, fitted with " Agla " micrometer attachment, was used to inject small volumes (0.005–0.01 ml.) of stimulating drug. Tests on isolated rabbit intestine were made in a 50-ml. organ bath. A light frontal writing isotonic lever was used, its total angle of excursion not exceeding 30 degrees.

Isolated lung.—Guinea-pig lung was used, the air passages being perfused with the medium described by McDowall and Thornton (1930). The animals were anaesthetized with ether to the point of respiratory arrest, and then killed by opening the thorax. Perfusion was made through an isolated heart cannula inserted into the trachea, fluid escaping through scarifications of the lung lobe. One lung only was perfused, the other being tied off at the bronchus. Preheating and perfusion were carried out by means of a mammalian heart perfusion apparatus. The temperature was maintained at 38° C. (±0.5°). Between each addition of drug, the lung was washed by forcing fluid through the alveoli under a pressure of 80 mm. Hg. For this purpose a 5-ml. " Record " syringe was attached to the upper end of the trachea by a glass T-piece. Records were made at a constant pressure of 40 mm. Hg. Antihistamine activity was

TABLE I

RO- COMPOUNDS 

Serial No.	R^1	R^2	Name of compound
3-0131	$CH_2CH_2N(C_2H_5)(C_2H_5)$	$CH_2CH_2N(CH_3)(CH_3)$	Diethylaminoethyl diphenyl-(dimethylaminoethoxy)-acetate
3-0190	$CH_2CH_2N(CH_3)(CH_3)$	$CH_2CH_2N(CH_3)(CH_3)$	Dimethylaminoethyl diphenyl-(dimethylaminoethoxy)-acetate
3-0255	CH_2CH_2N(morpholine)	CH_2CH_2N(morpholine)	β-morpholinoethyl diphenyl-(β′-morpholinoethoxy)-acetate
3-0275	$CH_2CH_2N(CH_3)(CH_2CH:CH_2)$	$CH_2CH_2N(CH_3)(CH_3)$	Allylmethylaminoethyl diphenyl-(dimethylaminoethoxy)-acetate
3-0257	$CH_2CH_2N(C_2H_5)(C_2H_5)$	CH_2CH_2N(morpholine)	Diethylaminoethyl diphenyl-(β-morpholinoethoxy)-acetate
3-0277	CH_2CH_2N(piperidine)	$CH_2CH_2N(CH_3)(CH_3)$	Piperidinoethyl diphenyl-(dimethylaminoethoxy)-acetate
3-0276	$CH_2CH_2N(CH_2CH:CH_2)(CH_2CH:CH_2)$	$CH_2CH_2N(CH_3)(CH_3)$	Diallylaminoethyl diphenyl-(dimethylaminoethoxy)-acetate
3-0280	CH_2CH_2N(morpholine)	$CH_2CH_2N(CH_3)(CH_3)$	β-morpholinoethyl diphenyl-(β-dimethylaminoethoxy)-acetate
3-0281	$CH(CH_3)CH_2N(C_2H_5)(C_2H_5)$	$CH_2CH_2N(CH_3)(CH_3)$	β-diethylamino*iso*propyl diphenyl-(β-dimethylamino-ethoxy)-acetate
3-0282	$CH_2CH_2N(CH_3)(CH(CH_3)_2)$	$CH_2CH_2N(CH_3)(CH_3)$	β-*iso*propylmethylaminoethyl diphenyl-(β-dimethylamino-ethoxy)-acetate
3-0289	$CH_2CH_2N(C_2H_5)(C_2H_5)$	$CH_2CH_2N(CH_3)(CH(CH_3)_2)$	Diethylaminoethyl diphenyl-(*iso*propylmethylamino-ethoxy)-acetate

expressed as percentage diminution of a standard histamine bronchospasm occurring in the presence of 0.5 mg. of each test compound.

Isolated heart.—Isolated rabbit hearts, perfused through the coronary vessels with Ringer-Locke solution at 38° C., were used.

Anaphylactic shock.—Guinea-pigs of varying age, weight, and sex were sensitized to egg-white as described by Landau, Marriott, and Gay (1948), and compounds were tested for their ability to protect them against anaphylactic shock.

General pharmacology.—Cats under chloralose anaesthesia were used in studying the effects of the drugs on blood pressure, spleen volume, and knee-jerk. Blood pressure was recorded from the carotid artery by mercury manometer and changes in spleen volume by plethysmograph and piston recorder. Knee-jerk was recorded by the method of Schweitzer and Wright (1937). Local anaesthetic activity was tested on the skin of the guinea-pig (Bülbring and Wajda, 1945); curariform activity on the isolated rat phrenic nerve-diaphragm preparation (Bülbring, 1946); analgesic activity in mice by the method described for rats by Davies, Raventos, and Walpole (1946); toxicity was determined by subcutaneous injection.

Assay of spasmolytic and antihistamine action.—Quantitative assays of spasmolytic activity were made on guinea-pig ileum and comparative activities against histamine, acetylcholine, potassium, and barium expressed in terms of the pA_2 scale (Schild, 1947). Schild's experimental procedure, however, was not applicable when potassium or barium was used as spasmogen, and was not therefore generally used for antihistamine and anti-acetylcholine assays, as it was considered essential to compare all forms of spasmolytic action on the basis of a common assay. The pA value was computed as $pA = -\log[A]$, where [A] is the molar concentration of antagonistic drug which produces a 50 per cent inhibition of a standard contraction induced by the spasmogen. A fixed time of contact (1 minute) was allowed between the introduction of the antagonist and of the spasmogen into the bath. Benadryl was used as standard antihistamine, atropine as standard anti-acetylcholine, and trasentin and papaverine as standards for anti-potassium and anti-barium activities respectively.

In the assay of antihistamine activity on the guinea-pig intestine, Schild's assay procedure was used for some of the compounds.

Results

Anti-acetylcholine activity

The compounds were all active in diminishing acetylcholine spasm in the guinea-pig ileum, though much less active than atropine. The results are shown in Table II, in which values for anti-acetylcholine activity measured at *p*H 8.0 are given in descending order. It was later found that the anti-acetylcholine activity of 3-0131 was very sensitive to alkali, storage of its solutions at *p*H 8.0 leading to rapid breakdown and inactivation. Its relative activity as recorded here was therefore not in accord with subsequent experiments. The tracings shown in Fig. 1 demonstrate yet another effect of *p*H upon the activity of 3-0131. It will be seen that, as *p*H diminished from 6.8 to 4.8, the same dose of compound produced an effect varying from slight to considerable inhibition of the acetylcholine spasm. Here the *p*H values refer to that of the drug solution *before* injection into the bath. The bath *p*H, after adding the drug solution, was not altered within the range of 0.1 *p*H unit. This effect was not related to storage, since the solutions were freshly prepared and were acidic, in which condition only very slow decomposition occurred.

TABLE II

ANTI-ACETYLCHOLINE ACTIVITY OF RO- COMPOUNDS COMPARED WITH ATROPINE ON GUINEA-PIG ILEUM

Ro- compound No.	Dose μg. ≡ 0.005 μg. atropine sulphate	Activity: atropine = 1.0
3-0190	2.0	0.0025
-0275	2.0	0.0025
-0277	3.0	0.0017
-0281	3.0	0.0017
-0289	3.0	0.0017
-0257	3.5	0.0014
-0276	4.0	0.0013
-0282	5.0	0.0010
-0131	13.0	0.0004
-0280	20.0	0.0003
-0265	>100.0	<0.0001

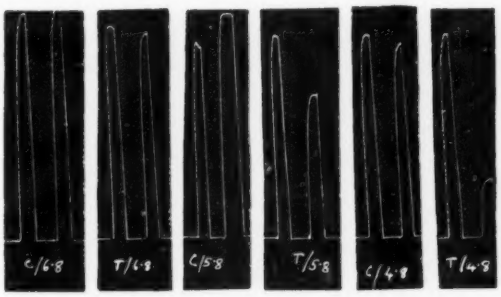


FIG. 1.—Effect of *p*H upon anti-acetylcholine action of Ro-0131. Read from left to right. In each group of two contractions, the first represents an ordinary acetylcholine contraction, the second an acetylcholine contraction in the presence of injected saline (controls marked "C") or in the presence of 5 μg. 3-0131 (tests marked "T"). Numbers refer to *p*H of the added solution.

pA values

Since it has been shown (Fig. 1) that spasmolytic activity in the compounds was greater when the *p*H of the solution was on the acid side, pA_2 values were determined with solutions of the hydrochlorides at *p*H 5.0–6.0. Doses of spasmogens were: acetylcholine, 0.1 μg. base; histamine, 0.1 μg. base; potassium chloride, 2.0 mg.; barium chloride, 1.0 mg. Results of these assays are given in Table III. All the Ro- compounds show some spasmolytic activity, including antihistamine action, on the isolated guinea-pig ileum. As a spasmolytic agent 3-0131 compares favourably with trasentin, but is a poor antihistamine compared with benadryl.

Antihistamine activity of the most active compounds was more accurately assayed by the method of Schild (1947). Results are given in Table IV, values for pA_2 representing the average of four determinations. All the Ro- compounds tested were considerably less active as antihistamines than benadryl and mepyramine. Trasentin, given a sufficiently long time of contact, is also more active as an antihistamine than the Ro- compounds tested similarly.

Further studies of antihistamine action

The values in Table V represent percentage reduction of histamine bronchospasm (due to 0.2 mg. histamine) produced by 0.5 mg. of each compound. The test substance in 0.05 ml. at *p*H 1.0 was introduced into the perfusion stream.

TABLE III

SPASMOLYTIC ACTIVITY OF RO- COMPOUNDS ON GUINEA-PIG ILEUM, COMPARED WITH TRASENTIN, BENADRYL, AND PAPAVERINE, EXPRESSED AS pA_2 UNITS

Compound No.	Anti-acetylcholine pA_2	Anti-histamine pA_2	Anti-potassium pA_2	Anti-barium pA_2
3-0131	6.2	5.7	4.8	4.6
-0190	6.0	5.1	4.5	4.1
-0265	4.8	3.8	4.0	3.8
-0275	6.0	5.0	4.8	5.0
-0257	5.7	4.6	4.6	4.3
-0277	6.1	5.5	4.7	5.1
-0276	5.6	5.4	5.0	4.7
-0280	4.8	4.5	4.1	4.0
-0281	5.8	5.4	5.0	4.7
-0282	6.0	5.0	4.4	4.4
-0289	5.9	5.4	4.9	4.3
Trasentin	5.9	5.4	4.4	4.5
Benadryl	5.7	7.4	4.3	4.5
Papaverine	—	—	—	3.9

TABLE IV

ANTIHISTAMINE ACTIVITY OF SOME RO- COMPOUNDS COMPARED WITH THAT OF BENADRYL, TRASENTIN, AND NEOANTERGAN USING SCHILD'S METHOD OF ASSAY

Compound No. or name	Time for maximum action (min.)	pA_2 (anti-histamine)
3-0131	5	6.9 ($\pm$0.1)
-0190	5	6.9 ($\pm$0.1)
-0275	10	5.7 ($\pm$0.2)
-0277	15	6.6 ($\pm$0.2)
-0276	10	6.0 ($\pm$0.4)
-0280	12	6.2 ($\pm$0.1)
-0282	7	5.9 ($\pm$0.1)
Trasentin	15	7.7 ($\pm$0.1)
Benadryl	11	8.6 ($\pm$0.1)
Neoantergan	7	9.8 ($\pm$0.1)

An equal volume of 0.1 N-HCl similarly injected did not affect the action of histamine on the lung. A tenth the quantity of benadryl (0.05 mg.) produced 67 per cent inhibition of the histamine effect; from this it has been calculated that benadryl is 7.6 times as active as 3-0131, the most active member of the series under test.

Further investigation of this compound was carried out on the guinea-pig in anaphylactic shock. The protection of guinea-pigs against death from anaphylactic shock was assessed for benadryl and for 3-0131 (Table VI). Though the number of animals used was small, it is concluded that 3-0131 is less active than benadryl in protecting guinea-pigs against anaphylactic shock. There are now available a number of antihistamines of greater activity than benadryl, so that 3-0131 may be classified as a weak antihistamine.

TABLE V

ANTIHISTAMINE ACTIVITY OF THE RO- COMPOUNDS AS ESTIMATED ON THE ISOLATED GUINEA-PIG LUNG

Ro- compound No. (in order of activity)	Percentage inhibition of histamine effect
3-0131	88 (±10)
-0277	59 (±10)
-0281	51 (±10)
-0276	47 (± 9)
-0289	46 (± 9)
-0190	25 (± 5)
-0282	24 (± 5)
-0280	23 (± 5)
-0275	No action
-0257	,, ,,
-0265	,, ,,

TABLE VI

COMPARISON OF RO-0131 WITH BENADRYL FOR ABILITY TO OPPOSE ANAPHYLACTIC SHOCK IN THE GUINEA-PIG

Compound	Dose in mg./kg. base	No. of animals shocked	No. of deaths	Percentage survival
None	—	10	9	10
Benadryl (as hydrochloride)	3	4	2	50
3-0131 (as hydrochloride)	3	4	4	0
3-0131 (as hydrochloride)	15	6	2	67
3-0131 (as salicylate)	15	10	3	70

TABLE VII

LOCAL ANAESTHETIC ACTIVITIES OF SOME RO- COMPOUNDS IN TERMS OF PROCAINE, ASSAYED BY GUINEA-PIG WEAL METHOD

Compound	Benadryl	Trasentin	3-0281	-0257	-0131	-0190	-0280	-0265	-0282
Local anaesthetic activity (procaine = 1) ..	0.68	0.86	>2.40	2.40	0.68	0.67	0.58	0.35	0.29

Local anaesthetic action

A representative number of the compounds were tested for local anaesthetic action, and the results are given in Table VII. The relative potencies were computed from dose-response curves. Since these were approximately parallel, the dose of each compound producing a median response (failure to respond to 18 out of 36 pricks) gave adequate comparison. Procaine was used as standard. All the compounds tested were local anaesthetics, 3-0281 and 3-0257 being more than twice as active as procaine.

Effects on blood pressure, spleen volume, and knee-jerk

Doses of 5 mg. of each compound injected into a cat under chloralose anaesthesia produced a steep but transient fall in blood pressure, which was not abolished by atropine. Trasentin and benadryl produced a similar effect. There were no significant effects on the knee-jerk response or on spleen volume. Blood pressure effects are shown in Table VIII. Benadryl differed from the Ro- compounds and trasentin in that it caused a transient fall in arterial pressure, followed by a sustained rise.

TABLE VIII

FALL IN BLOOD PRESSURE IN THE CAT (CHLORALOSE) DUE TO 2 MG./KG. OF EACH RO- COMPOUND INTRAVENOUSLY

Compound No.	B.P. fall in mm. Hg ± 10	Compound No.	B.P. fall in mm. Hg ± 10
3-0281, -0289	120	3-0190, -0282, -0276, -0257	50
-0131	80		
-0277, -0280	60	-0265	20

Effects on isolated rabbit heart

Compounds diminished the force of contraction of the isolated perfused rabbit heart, with no marked change in rate. The amplitude of the recorded beat greatly diminished, and cardiac action approached arrest in diastole. This effect was produced with relatively large amounts of each compound (0.5–1.0 mg.).

Effects on the isolated rabbit duodenum

The compounds arrested pendular movement and diminished tonus in the isolated rabbit duodenum. Concentrations of one part in 50,000 of all the Ro- compounds, excepting 3-0265, produced this effect.

Pharmacological tests with negative results

When tested for curariform activity, compounds 3-0131, -190, and -289 showed only a slight effect due to depression of the muscle itself. There was no demonstrable analgesic activity when compounds were injected subcutaneously into mice. When 3-0131 was combined with acetylsalicylic acid to form the corresponding salt, the analgesic activity of the product, injected intravenously into mice, was less than that of the acetylsalicylic acid alone.

Toxicity tests

The acute toxicity of 3-0131 was determined in some detail, as this drug was selected for clinical trial. The LD50 (subcutaneous in mice) was 475 mg./kg. Two other compounds—3-0190 and 3-0277—were tested elsewhere and found to be somewhat more toxic, the LD50 values being 300 and 350 mg./kg. respectively. Groups of 10 mice were used for each dose level, and log. dose-mortality curves were plotted. One half the LD50 of 3-0131, injected at intervals of 1–3 days over a period of 21 days, did not produce any signs of chronic toxicity.

Effects observed during administration of regular doses of 40 mg. three times daily in man for one week (two subjects) included nervousness, diminished appetite, and nausea, the latter after a period of 2–3 days. No other ill effects were observed. Single doses of 100 mg. were well tolerated by normal individuals.

In mice dying from the toxic action of this compound, the earliest signs were those of respiratory distress, with cyanosis visible about the mouth and nose. Very soon after there were convulsions and death would ensue, preceded by the animals leaping into the air. The trunk of the animal at death was usually in a state of extreme flexion, the mouth being wide open and jaws rigid. There was often marked exophthalmos and salivation. The general condition was suggestive of asphyxia, and toxic doses of the compound interfere with respiration in some manner that has not been determined.

Discussion

A series of compounds in which the chemical structure of benadryl and trasentin was combined has been found to possess similar pharmacological effects to these two drugs. The exact chemical hybrid of benadryl and trasentin (3-0131) shows a loss of specific antihistamine activity when compared with benadryl and a gain in general spasmolytic activity (anti-acetylcholine, anti-potassium, and anti-barium) by comparison with trasentin. The other homologues are in general less effective, either as antihistamines or as general spasmolytics.

The nature of the substituent on the nitrogen atoms does not greatly affect activity within the series, except in 3-0265. Here the presence of two morpholino-rings has rendered this compound less active than other members of the series, in all respects. More detailed study of 3-0131 has shown that this compound is unlikely to be of practical value purely as an antihistamine, but its low toxicity and high general spasmolytic activity make it worthy of clinical trial in allergy and in conditions involving smooth muscle spasm. The ability of the compound to lower arterial pressure in the cat, in contrast to benadryl, which raises arterial pressure, may prove to be a practical advantage if this difference holds true for man. Indications of the clinical value of 3-0131 have already been reported by Norman and Wrigley (1948).

On the basis of assays carried out on the guinea-pig gut, most of the compounds show higher anti-barium spasmolytic activity than papaverine. It is not, however, justifiable to argue from this that the Ro- compounds will be more useful than papaverine as spasmolytic drugs. Local anaesthetic activity is shared by these compounds in common with a number of other basic drugs. This is in accordance with the work of Burn and collaborators (Burn, 1948). Compounds 3-0257 and -281 are more active local anaesthetics than procaine. Curariform activity is absent, even with high concentrations of the drugs tested.

Summary

1. Eleven benzilic acid derivatives structurally related to benadryl and trasentin were found to possess varying degrees of general spasmolytic, antihistamine, and local anaesthetic properties. Their antihistamine activities were low compared with benadryl and mepyramine, but anti-acetylcholine activity was, in several compounds, higher than that of trasentin. Two compounds (3-0257 and 3-0281) are more potent local anaesthetics than procaine, being more than twice as active.

2. All compounds lower arterial blood pressure in the cat. The effect is not abolished by atropine.

3. The presence of a morpholino group in the molecule was found to diminish all types of pharmacological action, but no relationship of structure to activity was apparent for other substituents.

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A CINNOLINE COMPOUND ("528") FOR THE TREATMENT OF *TRYPANOSOMA CONGOLENSE* INFECTIONS

BY

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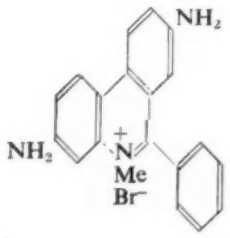
The most common pathogenic trypanosome of cattle throughout tropical Africa is *Trypanosoma congolense*. This species is relatively resistant to the arsenicals, diamidines and suramin, which are so useful against *T. gambiense*, the cause of the most common form of the disease in man. It was therefore not until compounds of the phenanthridinium group were introduced by Browning, Morgan, Robb, and Walls (1938) that the chemotherapy of *T. congolense* infections began to assume a favourable aspect. Of this chemical group "dimidium" bromide, 2 : 7-diamino-9-phenyl-10-methylphenanthridinium bromide (see formula I), first used in cattle by Carmichael and Bell (1944), has achieved considerable success in field practice; and another member of the group, 2 : 7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride, has recently been claimed by Brownlee, Goss, Goodwin, Woodbine, and Walls (1950) as an improvement on dimidium bromide in *T. congolense* infections in mice.

The first type of compound to be successful against *T. congolense* infections on a wide scale was therefore one in which the hetero-N is quaternized. This is interesting because no such type has found a place in the chemotherapy of human trypanosomiasis. It is a development that was foreshadowed many years earlier by the limited success of another group of quaternary ammonium compounds, viz., the styryl-quinolinium substances of Browning, Cohen, Ellingworth, and Gulbransen (1926). Quaternary nitrogen, this time at two points on the molecule, is again a feature of the most recent compound to be used successfully against *T. congolense* on a wide scale, namely "antrycide" (Curd and Davey, 1949, 1950), a salt of substituted quinaldinium-pyrimidinium residues connected by an imino linkage (see formula II).

Theoretical and Early Experimental Background

A preliminary note on the initial work which eventually led to "528" has already appeared (Keneford *et al.*, 1948). The work began before antrycide was introduced, and

* This work was started in the Warrington Yorke Department of Chemotherapy at the Liverpool School of Tropical Medicine. Its later chemical development was facilitated by the creation of a Medical Research Council Group for Research in Chemotherapy in the Department of Chemistry at Manchester University.



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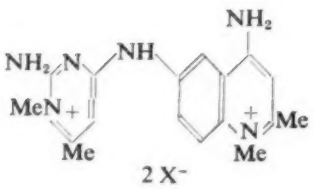

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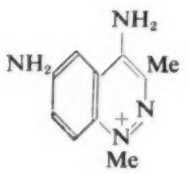

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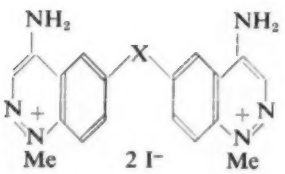

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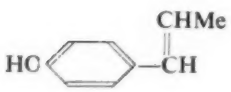

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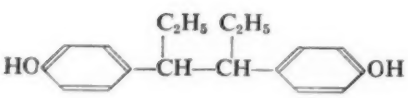

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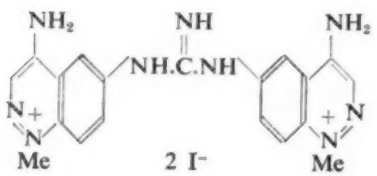

(VII)

two of the considerations by which it was influenced were (1) the probability that N-quaternization would favour activity against *T. congolense*, as testified by the success of the phenanthridinium compounds, and (2) the possibility that the phenanthridinium configuration might include redundant features, unnecessary for activity against *T. congolense*.

We therefore synthesized, and sought trypanocidal activity in, representatives of what might be regarded as a simplification of the phenanthridinium type, namely quaternary quinoline compounds, extending our studies at the same time to members of the closely related quaternary cinnolines and quinazolines. One obvious advantage of exploring these simplifications in preference to the original phenanthridinium type seemed to be that they might offer greater scope for introducing amino-groups into selected combinations of positions (amino-groups apparently being essential for trypanocidal activity in this class of compound) ; and we felt ourselves to be in a specially advantageous position for including cinnoline compounds in the investigation because of experience already gained with this group while examining its potentialities for the chemotherapy of malaria (Keneford and Simpson, 1947).

The chemical part of the work will be described elsewhere, together with notes on the degree of trypanocidal activity observed in individual compounds. Here it will suffice to give no more than an outline of the steps leading towards " 528," with a detailed consideration of the results obtained with that substance.

The first few pure preparations of the mono- and diamino-quaternary quinolines, cinnolines, and quinazolines examined showed little or no activity. An important clue was, however, afforded by finding that one of these compounds, though inactive in the pure state, was significantly active when tested as a crude preparation before being finally crystallized. This was 4 : 6-diamino-1 : 3-dimethylcinnolinium iodide (formula III). The impure (and trypanocidally active) preparation of this substance was a crude reduction product of the corresponding 6-nitro salt, an intermediate in one of the routes of synthesis towards the eventual 6-amino compound. Since a pure preparation of this nitro-analogue was also devoid of trypanocidal activity, the obvious interpretation was that the activity observed in the impure preparation must, in fact, have been a property of one of the impurities rather than of the actual compound towards which the synthesis was directed. It seemed that the hypothetical trypanocidal impurity might indeed be highly active, since the crude preparation of which it necessarily formed only a fraction was capable of clearing trypanosomes from the blood of infected mice not only temporarily but permanently. The strain of trypanosome used for this early work was Strain II of Browning and Calver (1943), which had been maintained in mice for many years in Liverpool, and which normally produced acute infections ending fatally about five to seven days after inoculation. For preliminary evaluation of trypanocidal activity the drugs were given in a single dose intraperitoneally, on a twofold dosage-scale, in aqueous solution in 0.5 ml. per 20 g. body weight ; and the very rough measures of activity used (admittedly without any pretensions to precision, though very useful for indicating a compound's potentialities) were the Maximum Tolerated Dose, the highest dose which failed to kill any of five mice, and the Minimum Curative Dose, the lowest dose which cured at least four of five mice. The very approximate therapeutic index (MTD/MCD) obtained from these values was about 2 for the most active of the impure preparations tested. Bearing in mind that the impurity formed only a part of the preparation tested, this figure suggested a high degree of activity in the hypothetical impurity, because the therapeutic indices obtained in the same way for presumably pure specimens of the two most active of the phenanthridinium compounds known at that time, dimidium bromide and " phenidium " chloride (Browning *et al.*, 1938), were only 15 and 3 respectively.

It seemed possible that the hypothetical impurity responsible for trypanocidal activity in the crude preparation of III might be a symmetrical binuclear compound of the type shown in formula IV, with CH_3 in positions 3 and 3′, formed as a side-reaction during conversion of the nitro- to the amino-quaternary salts. This seemed to be a possibility not only for chemical reasons, the likelihood being that the linkage *X* was the azo group -N : N-, but also because of the known composition of certain non-arsenical trypanocidal substances Examples are trypan red, suramin, the aromatic diamidines, and bis-quinolyl compounds such as Surfen C (Iensch, 1937) and its analogues. Compounds conforming to formula IV would have molecular weights of the same general order as those of the substances named, and these weights would therefore satisfy the hypothetical requirement of falling within a narrow critical range which might be necessary for trypanocidal activity. Perhaps more significantly, compounds of formula IV would share also with the trypanocides named the general pattern of two identical, or nearly identical, substituted cyclic units with a simple connecting linkage. A further chemical analogy, not known at the time, became available with the announcement of antrycide (Curd and Davey, 1949) after our original working hypothesis had been published (Keneford *et al.*, 1948). Antrycide also, as shown in formula II, consists of two cyclic residues joined by a simple linkage, but in this case the cyclic components are not identically constituted as in the trypanocides just mentioned. The feature they do share with our envisaged type (formula IV) and not shown by the other trypanocides is bis-quaternization, to which attention has already been drawn in our opening paragraphs.

The variety of these analogies strengthened the case for examining a range of compounds of type IV with linkages of various other types as well as the azo, which chemical reasons suggested as the most likely one in the active impurity of the crude compound III. An obvious further extension of the investigation would be to examine a similar range of bis-quinolinium and related symmetrical heterocyclic compounds.

There are interesting analogies not only between the chemical structure of our envisaged type and that of known trypanocides, but also between the sequence of discovery leading to " 528 " and that which led to certain other therapeutic substances. Both in our own work and in an entirely different field, that of the search for synthetic sex hormones, the sequence was: (i) Exploration of simplifications of a relatively complex chemical configuration known to be associated with biological activity ; (ii) activity found to be present in a crude preparation of one of these simple derivatives but absent from pure specimens ; and (iii) identification of the active impurity as a member of a chemical type in which the cyclic feature of the pure substance is duplicated to give a substance of symmetrical structure.

In the work leading to " 528," the relatively complex configuration of stage (i) was the phenanthridine ring-system (formula I) ; and the mono- and bis-compounds of stages (ii) and (iii) were those of formulae III and IV (though we have not yet reached the stage of isolating and characterizing the actual impurity). In the work leading to synthetic sex hormones by Dodds *et al.* (1939) and Campbell *et al.* (1938, 1939), the complex structure of (i) was the naturally occurring *cyclo*pentenophenanthrene configuration ; and the mono- and bis-compounds of stages (ii) and (iii) were those shown in formulae V and VI.

The steps leading to " 528," an early outline of which was already given in our preliminary note (Keneford *et al.*, 1948), also found some analogy with those leading to antrycide, later described by Curd and Davey (1950). Here also trypanocidal activity was found in a crude but not in a pure precursor of the eventual trypanocidal compound. However, the precursor was not a mono-analogue of the eventual bis-substance, but a bis-compound already conforming to the antrycide structure, but with only one of the N-atoms quaternized. The active impurity was thought to be a substance similar to

the compound for which the synthesis was intended but with a nitrogen atom quaternized in both the ring systems, and this clue led directly to the antrycide molecule.

Bis-Cinnolinium and Bis-Quinolinium Compounds

The first bis-3-methylcinnolinium compounds of type IV to be synthesized and examined were those with guanidine, urea, and thiourea linkages. These proved to be quite inactive in maximum tolerated subcutaneous dosages of about 0.25, 4, and 8 mg./20 g., respectively. This was discouraging, but was obviously not enough to justify abandoning the symmetrical type of compound. One reason why our faith in compounds of that type was not seriously shaken was that we had not yet synthesized and examined the azo-substance, which we had originally suspected of being the active impurity in the crude preparation of III. Unexpected difficulties arose in synthesizing this substance, and, pending solution of these difficulties, attention was concentrated on a series of mono- and bis-compounds unmethylated in the 3- position. Here the surprising development arose among the mono-cinnolinium substances that, while pure specimens of 4 : 6-diaminocinnolinium iodide were quite inactive, unequivocally pure specimens of its 6-nitro precursor were curative at the maximum tolerated dose of about 0.75 mg./20 g. subcutaneously. This seemed to reopen the possibility of finding useful substances among those of the simpler mono-structure, and examination of these is proceeding, but our main energies continued to be directed towards the bis-substances.

The bis-compounds of type IV, unmethylated in position 3-, which then engaged our main attention might be described as N_6-linked bis-4:6-diaminocinnolinium substances and their corresponding bis-quinolinium analogues. The quinolinium salts synthesized and examined were iodides or chlorides with azo, urea, thiourea, and guanidine linkages. The maximum tolerated subcutaneous doses of the azo compound in mice was about 0.125 mg./20 g. body weight, and about half this was capable of temporarily clearing trypanosomes from the blood. The urea compound was tolerated in doses of about 1.0 mg./20 g., but it was only at this relatively high dosage-level that the blood was temporarily cleared in a few of the mice thus treated. The maximum tolerated dose of each of the other two quinolinium substances, like that of the azo compound, was about 0.125 mg., but they showed no trypanocidal activity at that dosage.

These few bis-quinolinium substances were therefore relatively toxic and ineffective as a group. The azo compound was more active than the other derivatives, and this vindicated in some measure our belief that the active impurity in the crude preparation which first directed our attention to these bis-substances might have been an azo-linked compound. However, the azo-bis-cinnolinium derivative in the present series, as in the 3-methyl series above, also proved unexpectedly difficult to synthesize, and chemotherapeutic observations on this substance must await a later communication. The urea compound was found to clear the blood temporarily but not permanently in maximum tolerated doses of about 1.0 mg./20 g. body weight ; and the thiourea was inactive at maximum doses of about 0.5 mg./20 g. But with the guanidine compound the activity observed was of a sufficiently high order to demand precise definition. This is the substance which, for convenience, we designate as " 528."

N^1 : N^3-BIS(4′-AMINOCINNOLYL-6′)-GUANIDINE DIMETHIODIDE (" 528 ")

The appropriate chemical designation of 528 appears at the head of this paragraph, and its formula is VII. It is a yellow crystalline substance, fairly soluble in water, giving solutions which are also yellow in colour.

In a series of experiments the toxic and therapeutic actions of 528 in mice were compared with those of antrycide methylsulphate. Each drug was given subcutaneously, in aqueous solution, in a single dose per mouse and in a volume of 0.4 ml. per 20 g. body weight. For the toxicity tests uninfected mice were used. For therapeutic tests the trypanosome strain used was the Busimbi *T. congolense* of Curd and Davey (1949, 1950), kindly sent to us by Dr. Davey. This produced acute fatal infections similar to those caused by the strain on which our earlier observations had been made. Mice were inoculated intraperitoneally so that 1 to 20 trypanosomes per microscope field could be seen in thin blood films two or three days later, at which stage they were treated.

TABLE

RESULTS OF TESTS WITH " 528 " AND ANTRYCIDE METHYLSULPHATE. TOXICITY IN UNINFECTED MICE AND THERAPEUTIC ACTIVITY IN MICE INFECTED WITH *Trypanosoma congolense*

Compound	Dose (mg./20 g.)	No. treated	Result	Log dose probit regression slope (*b*)	Lethal doses (mg./20 g.)		Curative doses (mg./20 g.)		Therapeutic indices (with 95% fiducial limits)	
					LD50	LD10	CD50	CD90	LD50/CD50	LD10/CD90
"528"			% killed							
	2.20	40	67							
	1.69	48	52	5.42	1.73	1.03				
	1.30	43	23							
	1.00	23	9						22.9	11.6
			% cured						(20.5–25.5)	(9.6–13.9)
	0.123	21	100							
	0.095	22	95	18.02			0.076	0.089		
	0.073	21	43							
	0.062	26	4							
Antrycide			% killed							
	1.10	45	78	6.07	0.82	0.49				
	0.84	83	55							
	0.75	60	37							
	0.65	52	29						33.4	12.5
			% cured						(30.0–37.3)	(10.1–15.6)
	0.042	20	100							
	0.032	20	75							
	0.031	36	72				0.024	0.039		
	0.025	22	41	6.40						
	0.019	22	36							
	0.016	36	11							

Toxicity was measured by percentage mortalities at different doses. The deaths occurred within about six hours of injection, usually within the first hour, both after antrycide and after 528. The two drugs were also alike in producing similar signs of toxic action before death, as briefly described elsewhere (Lourie and Walker, 1951). In the therapeutic tests, mice in which the peripheral blood remained free of trypanosomes for 30 days after treatment were regarded as cured.

The results of these tests for toxicity and therapeutic action are set out in the Table. The subcutaneous LD50 for antrycide was found to be considerably higher than those reported by Curd and Davey (1949, 1950) and by Goodwin and Walls (1950), our figure being 0.82 mg./20 g., while Curd and Davey quote 0.4 to 0.5 and Goodwin and Walls 0.46 mg./20 g. body weight, administered in volumes of unspecified size. It is notorious that small differences of technique may lead to wide differences in the results of tests for toxicity or for therapeutic action. We have examined one particular example of this in respect of antrycide methylsulphate, without being in a position to say whether it has contributed to the discrepancies between our own toxicity figures and those of the other workers. This is that the lethal effect of a subcutaneous dose of antrycide methylsulphate (and probably of any other drug which kills quickly) is largely dependent on the volume of the dose injected. All our own injections were given in a volume of 0.4 ml. per 20 g. body weight, and it seems specially advisable to quote the dose-volume (or drug concentration) whenever precise figures are given for the lethal action of any drug that kills rapidly. This point is elaborated in a separate communication (Lourie and Walker, 1951).

The Table shows that both the lethal and the curative doses of 528 are higher than those of antrycide, and in very roughly the same proportions for the curative as for the lethal doses. The effect is that the differences between the therapeutic indices of the two drugs are slight. When the indices compared are those given by LD50/CD50 they are 22.9 for 528 and 33.4 for antrycide, i.e., giving a ratio of 1.46 in favour of antrycide. Calculation of fiducial limits to these particular indices shows that this slight advantage in favour of antrycide is statistically significant.

The 50 per cent end-points are rightly much favoured for the statistical definition of biological properties, because their standard errors are less than those of any other end-points that might be chosen (Trevan, 1927). They suffer from the disadvantage, however, that for practical chemotherapy the doses which kill 50 per cent of subjects and which cure only 50 per cent are very far from the ideal. Doses required in practice are those which kill practically none and cure practically all; but the figures representing doses (such as the LD1 and the CD99) which approach those ideal requirements have such large standard errors that very little reliance can be placed on them. A compromise is needed between the accuracy coupled with therapeutic undesirability of the 50 per cent end-points, on the one hand, and the inaccuracy coupled with therapeutic virtues of the LD1 and CD99 on the other. This compromise may be struck by the LD10 and CD90, and the Table shows that in the therapeutic indices derived from those doses (i.e., the indices given by LD10/CD90) the slight superiority of antrycide over 528 is lost and the two compounds emerge as approximately equal in value, their indices now being 11.6 and 12.5. (It is the very steep slope of *b* for cures with 528 which redresses for that drug the less favourable relationship to antrycide which it assumed when the therapeutic indices were derived from 50 per cent end-points.)

The close agreement between the therapeutic indices for 528 and for antrycide methylsulphate in mice clearly justifies a trial of this new compound for *T. congolense* infections in cattle. As for its potentialities against other trypanosome species, we have made a few provisional tests with an old laboratory strain of *T. rhodesiense*. The tests have shown that mice infected with this strain may be cured by the same doses of 528 as are curative for *T. congolense* infections. In the present studies we have, however, concentrated on *T. congolense* rather than on *T. rhodesiense* because, for many reasons, field workers in Africa still regard the control of cattle trypanosomiasis as a more pressing and difficult problem than that of human trypanosomiasis.

Summary

1. Trypanocidal activity was sought in representatives of what might be regarded as a simplification of the phenanthridinium type, namely, quaternary quinoline compounds, and in members of the closely related quaternary cinnolines and quinazolines.

2. The trypanocidal activity detected in crude preparations of one particular cinnolinium salt was lost when pure specimens of the same substance were examined. It was therefore inferred that the trypanocidal activity found in the crude preparations must have been a property of one of the impurities present in those preparations.

3. On both chemical and biological grounds it was thought likely that the active impurity might be a substance with two cinnolinium residues joined by a simple linkage. A series of such substances was therefore synthesized and examined, and some of them were in fact found to be trypanocidally active. The most active was the compound $N^1 : N^3$-bis(4′-aminocinnolyl-6′)-guanidine dimethiodide which, for convenience, we designate as " 528."

4. When tested against *T. congolense* infections in mice, the therapeutic index, LD10/CD90, of 528 was found not to differ significantly from that of antrycide methylsulphate.

We have greatly benefited by consultation with Dr. D. J. Finney, Lecturer in the Design and Analysis of Scientific Experiment in the University of Oxford, and we are indebted to him also for calculating the statistical values shown on the Table.

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THE EFFECT OF COCAINE AND CHRONIC SYMPATHETIC DENERVATION OF THE HEART ON THE CHRONOTROPIC ACTION OF ADRENALINE AND NORADRENALINE

BY

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From observations published during the last year or so, it would appear that post-ganglionic sympathetic denervation usually potentiates the response of effector organs to noradrenaline more than their response to adrenaline. This has been shown for the nictitating membrane of the cat by Bülbring and Burn (1949) and Lockett (1950a), and for the pupil dilator muscle of the cat by Burn and Hutcheon (1949). Lockett (1950b) found that in dogs thoracic and lumbar sympathectomy on the one hand potentiated the pressor response to noradrenaline more than that to adrenaline, but, on the other, increased the chronotropic action of adrenaline more than of noradrenaline.

Although it is known that cocaine sensitizes the nictitating membrane and increases the pressor response not only to adrenaline but also to noradrenaline (cf. Euler, 1950), no quantitative comparisons are available. The chronotropic action of adrenaline appears to be only slightly affected by cocaine. Rosenblueth and Schlossberg (1931) found no effect of cocaine on the response of the denervated cat's heart to adrenaline, while Peralta and Lizarralde (1946) found that there was no effect on the maximal increase in heart rate but that the return to the basal value was retarded. In a brief note, Innes and Kosterlitz (1950) reported that cocaine potentiated the chronotropic action of noradrenaline but not of adrenaline.

METHODS

Operative.—All but five cats were subjected to a preliminary operation under nembutal-ether anaesthesia, in which the right adrenal was removed and the left adrenal medulla denervated by left lumbar sympathectomy and section of the left splanchnic nerves. Usually two to three weeks were allowed for recovery from this operation. Both adrenals of the remaining five cats were removed during the acute experiments.

When the heart was to be deprived of part or the whole of its sympathetic nerve supply before the acute experiment, the following technique was adopted. For chronic removal of the stellate ganglia, both ganglia were removed in a one-stage operation under nembutal-ether anaesthesia by the antero-lateral approach through the first intercostal spaces. Great care had to be taken to avoid causing a pneumothorax by injury to the pleura. For complete chronic sympathetic denervation, the middle cervical ganglia were removed through a midline incision in the neck during the preliminary operation for inactivation of the adrenals. The stellate ganglia and the thoracic ganglia down to T 8 were removed on both sides at a single operation, the stellate ganglia as

already described and the remaining ganglia through the fifth or sixth intercostal spaces. Before closure of the intercostal gap the lungs were maximally inflated in order to reduce the pneumothorax to a minimum. Strict asepsis was observed. Penicillin was injected twice daily for the first two to three post-operative days.

Experimental.—In cats whose hearts had been partly or completely deprived of their sympathetic nerve supply in a preliminary operation, only the vagi remained to be cut in the acute experiment before testing the actions of adrenaline and noradrenaline. For acute denervation, the vagi were cut and then either the middle cervical and stellate ganglia removed or the middle cervical, stellate, and thoracic ganglia down to T 8. The anaesthetics used in the acute experiments were either nembutal (45 mg./kg. body weight), chloralose (0.05 g./kg.)-urethane (0.5 g./kg.), or ether. A few experiments were performed in the spinal preparation one hour after decapitation under ether.

The heart rate was counted on B.P. tracings obtained from the femoral artery with a mercury manometer. The heart rate (beats/min.) during the 30 sec. before the injection of the drugs was taken as the basal heart rate. After the injection, the heart rate was generally counted for 90 sec. and the response given as the mean increase of heart rate (beats/min.) during this period. The responses of the heart to sympathomimetic drugs may differ either in the peak reached, in the duration of the response, or, as is most often found, in both peak and duration. The procedure adopted was considered to be a fairly satisfactory compromise between what was required to characterize the chronotropic responses and the amount of labour involved in counting the heart rate from the B.P. tracings. In a number of experiments the heart rates were counted for 2 min. instead of 1½ min., but this did not lead to a different interpretation of the results. The best method would have been a mechanical counter permitting continuous counting. Attempts have been made by us to use the QRS deflection of the e.c.g. for this purpose. For several reasons, however, the counting from the B.P. tracing proved to be the more reliable of the two methods.

l-Adrenaline (B.D.H.) was dissolved in 0.01 N-HCl and *l*-noradrenaline D-bitartrate monohydrate (Sterling-Winthrop Institute) in water, both in a concentration of 1 mg. base/ml. These stock solutions were kept at $-5°$ C. The final dilutions were made immediately before the experiment with Ringer-Locke solution containing ascorbic acid (1 mg./ml.). The drugs were injected into the femoral vein by means of a recalibrated tuberculin syringe. The volumes injected varied between 0.1 and 0.4 ml., and the time taken for the injection was always 15 sec. The quantities injected are given as the free bases.

Care was taken to maintain the temperature of the cats as constant as possible. The variations did not exceed 1° C. and were usually of the order of 0.5° C. Within these limits, no correlation was found between changes in body temperature, basal heart rates, and chronotropic responses to adrenaline and noradrenaline.

The results were examined statistically by Mr. M. H. Quenouille and Mr. F. H. C. Marriott, who used the methods of R. A. Fisher (Quenouille, 1950). The significance of a difference is expressed as the probability P of an observed difference occurring at random in a homogeneous population.

RESULTS

1. *The chronotropic actions of* l*-adrenaline and* l*-noradrenaline on hearts with intact sympathetic nerve supply*

It is a well-known fact that, in the intact animal, the chronotropic actions of adrenaline and noradrenaline are modified reflexly by the changes in arterial B.P., resulting with noradrenaline in a considerable bradycardia. For this reason an attempt was made to study the chronotropic actions of adrenaline and noradrenaline

after section of the vagus and sinus nerves. It was found, however, that with the sympathetic tone unrestrained by the buffer nerves the basal heart rate was very high (280 beats/min.) and small doses of adrenaline and noradrenaline had no or little effect. After removal of both stellate ganglia, the basal heart rate dropped to a mean of 183 beats/min. and measurable chronotropic responses were obtained.

It thus became obvious that the vasomotor centre had to be excluded, which was done by testing the chronotropic responses in the decapitate preparation. In each of four cats the responses to noradrenaline were smaller than to adrenaline (0.8–1.6μg.). Severing the preganglionic fibres to the stellate ganglia caused little change, as also did removal of the stellate ganglia. In the latter case, the mean increase after 1.6 μg. of either drug was greater by four beats/min. than before or after section of the preganglionic fibres ; this difference was statistically significant ($P<0.05$). The responses to adrenaline remained greater than those to noradrenaline. There was a tendency for the basal heart rate to be lower after removal of the stellate ganglia (177 beats/min.) than before and after section of the preganglionic fibres to the ganglia (199 and 190 beats/min. respectively), but this effect was very much smaller than that described above for the anaesthetized cat with buffer nerves cut.

2. *The effects of cocaine and chronic sympathetic denervation on the chronotropic action of* l-*adrenaline and* l-*noradrenaline*

Results were obtained on 19 cats with acute removal of the stellate and middle cervical ganglia, 5 cats with complete acute denervation of the heart, 10 cats with chronic removal of the stellate ganglia, and 2 cats with complete chronic sympathetic denervation. In all cats the vagi were divided acutely.

Before the administration of cocaine, the mean basal heart rate was 164 in the cats with acute removal of the stellate ganglia, 165 in those with complete acute denervation, and 173 in those with chronic removal of the stellate ganglia or chronic complete sympathetic denervation. These differences were not significant. The change in basal heart rate after 8 mg. cocaine hydrochloride/kg. body weight was $+18$ in the experiments with acutely denervated hearts and -1 in those with chronically denervated hearts. Thus, cocaine significantly increased the heart rate in cats with acutely denervated hearts, 18 ± 4 ($P<0.001$) ; the difference between the cocaine effects on the acutely and chronically denervated hearts was also significant, 19 ± 8 ($P<0.05$).

A qualitative analysis of the effects of cocaine and of chronic sympathetic denervation on the chronotropic actions of adrenaline and noradrenaline is given in Tables I and II. Each cat received 2 to 3 different doses (0.4–2 μg.) of the drugs before and after the intramuscular injection of cocaine hydrochloride (8 mg./kg.). In cats with acutely denervated hearts adrenaline was generally more effective than noradrenaline, and in cats with chronically denervated hearts the reverse holds. After cocaine was given to cats with acutely denervated hearts, noradrenaline was more effective than adrenaline. The effect of cocaine on the response to adrenaline in cats with acutely denervated hearts was not consistent, while in those with chronically denervated hearts a depression occurred significantly more often than a potentiation ($P<0.05$). With noradrenaline, given to cats with acutely denervated hearts, potentiation by cocaine was preponderant ($P<0.001$), whereas in the animals with chronically denervated hearts no consistent effect could be observed.

TABLE I

Comparison of the chronotropic responses, before and after cocaine, of cats with acute or chronic removal of the stellate ganglia or complete sympathetic denervation of the heart. The vagi were cut acutely*

Treatment	Number of cats in which the chronotropic responses were :	
	Adrenaline > noradrenaline	Noradrenaline > adrenaline
Acute denervation:		
Before cocaine	13	3
After cocaine	0	16
Chronic denervation:		
Before cocaine	1	11
After cocaine	0	12

* The results of 8 exps. in which the doses of adrenaline and noradrenaline were not identical have been omitted from this table.

TABLE II

Effect of cocaine on the chronotropic responses to adrenaline and noradrenaline of cats with acute or chronic removal of the stellate ganglia or complete sympathetic denervation of the heart. The vagi were cut acutely. Figures indicate the number of cats in each category

Effect of cocaine	Adrenaline		Noradrenaline	
	Acutely Denervated hearts	Chronically Denervated hearts	Acutely Denervated hearts	Chronically Denervated hearts
Potentiation	3	1	21	5
Depression	10	8	2	5
Doubtful*	11	3	1	2

* A mean difference of less than ± 3 beats/min. was considered doubtful.

The anaesthetic appeared to have had little or no influence, since similar results were obtained in cats anaesthetized with nembutal or chloralose-urethane and in the non-anaesthetized decapitate preparation. Further, differences in basal heart rate had no obvious effects on the chronotropic responses.

The examination of the dose-response relationship showed that the logarithms of the increases in heart rate varied linearly with the logarithms of the doses of adrenaline and noradrenaline. The regression coefficients (Table III) for log increase in heart rate on log dose varied only slightly between the different groups, adrenaline before and after cocaine, and noradrenaline before and after cocaine. The geometric means of the responses confirmed that the chronotropic action of noradrenaline was increased by cocaine in the acutely but not in the chronically denervated cats and, also, that chronic sympathetic denervation had a potentiating effect. The chronotropic responses to adrenaline were not affected by the different operative treatments.

It appeared to be of interest to examine the effect of cocaine on the chronotropic response of hearts with intact sympathetic innervation. In 4 decapitate cats, cocaine hydrochloride (8 mg./kg.) potentiated the response to noradrenaline very

TABLE III

EFFECT OF DIFFERENT OPERATIVE TREATMENTS ON CHRONOTROPIC RESPONSES

Operative treatment	Geometric means of increases in heart rate (beats/min.) calculated for			
	1 μg. *l*-adrenaline		1 μg. *l*-noradrenaline	
	Before Cocaine	After Cocaine	Before Cocaine	After Cocaine
Acute removal of stellate and middle cervical ganglia and section of vagi (19 cats)	21	17	13	30
Acute denervation of heart (5 cats)	21	20	16	37
Removal of stellate ganglia at least 7 days previously and acute section of vagi (10 cats)	19	12	27	29
Sympathetic denervation of heart 8–9 days previously and acute section of vagi (2 cats)	25	19	60	60
Regression coefficients with S.E. (log increase in heart rate on log dose)	0.69 ±0.05	0.82 ±0.07	0.76 ±0.07	0.59 ±0.04

With adrenaline, whether before or after cocaine, the effects of different operative treatments were not significantly different. With noradrenaline, before cocaine, the responses were significantly lower in the acutely operated than in the chronically operated animals ($P<0.01$). With noradrenaline after cocaine, there was no difference between the acutely and chronically operated cats; complete chronic sympathetic denervation led to greater responses than chronic removal of the stellate ganglia ($P<0.05$).

markedly (+52, 35, 18, 55 beats/min.), while, with the exception of one cat, the response to adrenaline was practically unaffected (+4, 2, 2, 24 beats/min.).

Since the rise of arterial B.P. was much greater after noradrenaline than after adrenaline, it was necessary to test the possible effects of variations in arterial B.P. on chronotropic responses. For this reason, an experiment was designed in which the chronotropic responses and the changes in arterial B.P. were expected not to run parallel (Fig. 1). Cocaine potentiated the chronotropic responses to noradrenaline; these responses were not further influenced by tetraethylammonium chloride or by dibenamine. On the other hand, the rise in arterial B.P. after injection of noradrenaline was potentiated by both cocaine and tetraethylammonium chloride and then almost completely abolished by dibenamine. It is further to be noted that the basal level of B.P. was very much lower after dibenamine than in the early stages of the experiment, and thus changes in basal B.P. appear to have very little influence on the chronotropic responses.

Discussion

The main conclusions which may be derived from the reported results are that cocaine and chronic postganglionic sympathetic denervation potentiate the chronotropic action of noradrenaline but not that of adrenaline.

If a similar effect did obtain for adrenaline, it was too small to be demonstrable with the methods employed by us. There is general agreement that cocaine does not increase the peak of cardiac acceleration reached after adrenaline injection (Rosenblueth and Schlossberg, 1931; Peralta and Lizarralde, 1946; present results). As to the prolongation of the adrenaline action by cocaine reported by Peralta and

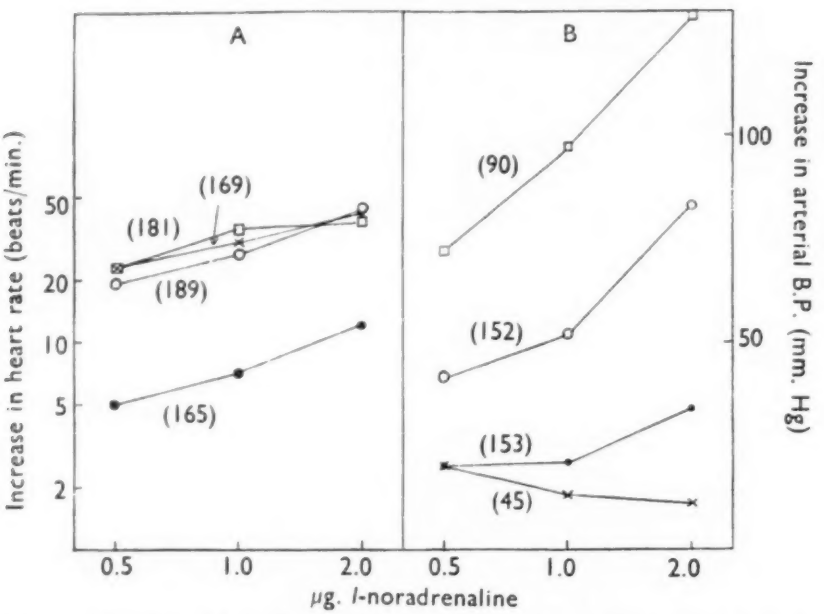

FIG. 1.—Cat, anaesthetized with nembutal (45 mg./kg.), both adrenals, stellate and middle cervical ganglia acutely removed. Increase in (A) heart rate and (B) arterial B.P. during first 1½ min. after injection of *l*-noradrenaline; before cocaine ●——●; after cocaine hydrochloride (8 mg./kg.) ○——○; cocaine followed by tetraethylammonium chloride (30 mg./kg.) □——□; and dibenamine (15 mg./kg.) ×——×. The mean values of basal heart rate and arterial B.P. are given in parentheses.

Lizarralde (1946), these authors used considerably larger doses of adrenaline than were employed in the present investigation. It is possible that a slight prolongation remained unnoticed by us, as we counted the heart rate for only 90 sec. after injection of adrenaline. With regard to the effects of chronic denervation, Burrett (1940–1) denervated the hearts of cats and followed up for each individual animal the maximal increase in heart rate after injection of adrenaline at varying intervals after denervation. The increase in sensitivity found by this author was not detectable by us. This may be due to differences in technique: at the operation for denervation Burrett not only removed the sympathetic nerve supply to the heart but also cut the vagal fibres, whereas we divided the vagi immediately before testing the chronotropic responses. Further, we inactivated the adrenals, while Burrett did not. Finally, it was inherent in the design of our experiments that we had to compare the effects of acute and chronic denervation in different cats, which made the evaluation of small effects impossible.

Lockett (1950b) found that in bitches chronic thoracic and lumbar sympathectomy increased the chronotropic action of adrenaline more than that of noradrenaline. The tests were made while the dogs were asleep and had been given atropine. Although the experimental procedure differed thus in several ways from ours, the possibility of a species difference will have to be kept in mind.

No detailed examination has so far been made to determine the relationship between duration of postganglionic sympathetic denervation and potentiation of the

chronotropic response to noradrenaline. The fact that in a small number of cats with acute removal of the stellate ganglia (three experiments) the responses to noradrenaline were fully potentiated before the administration of cocaine, i.e., within one or two hours after denervation, seems to suggest that the process of potentiation may be very rapid.

From the evidence so far available, it is difficult to offer an explanation for the rise in basal heart rate which is found when cocaine is given to cats with acutely denervated hearts, but which is absent in cats with chronically denervated hearts. The phenomenon may possible be due to a sensitization by cocaine of the acutely denervated heart to the small quantities of noradrenaline present in the circulating blood.

The analysis of the phenomena described is still incomplete, and an attempt to formulate a hypothesis would be premature. There is, however, one fact which will have to be embodied in any such hypothesis, viz. that, with or without previous interruption of the sympathetic nerve supply, cocaine instantaneously causes a potentiation of the chronotropic action of noradrenaline similar to that found after chronic sympathetic denervation. It is of further interest to note that the enhancement of the chronotropic responses to adrenaline and noradrenaline appears in principle to obey the same laws as found in the sensitization of the pupil and the nictitating membrane to these drugs (Bülbring and Burn, 1949 ; Burn and Hutcheon, 1949 ; Lockett, 1950a).

Summary

1. In the spinal cat, either before or after acute section of the preganglionic fibres to the stellate ganglia or acute removal of the ganglia, the cardio-accelerator response to *l*-adrenaline was usually greater than to *l*-noradrenaline (0.4–2.0 μg.).

2. No significant differences were found in the basal heart rates of cats with acute or chronic removal of the stellate ganglia or chronic sympathetic denervation. In cats with acutely denervated hearts, cocaine hydrochloride (8 mg./kg.) raised the basal heart rate.

3. In cats with acutely denervated hearts, adrenaline generally had a greater cardio-accelerator effect than noradrenaline, while in cats with chronic sympathetic denervation of the heart noradrenaline was always more effective than adrenaline. Cocaine potentiated the chronotropic response to noradrenaline in cats with acutely denervated hearts. Cocaine had no significant effect on the response to noradrenaline in cats with chronic sympathetic denervation of the heart and to adrenaline in cats with acutely denervated hearts, while it depressed the adrenaline response in cats with chronic sympathetic denervation of the heart. No consistent difference was found between cats with complete sympathetic denervation of the heart and those with removal of the stellate ganglia only.

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THE ABSENCE OF AN EFFECT OF DIETARY PROTEIN ON THE LIBERATION OF LIVER SYMPATHIN AFTER STIMULATION OF THE HEPATIC NERVES

BY

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When Cannon and Uridil (1921) first showed that stimulation of the hepatic nerves liberated sympathin from the liver into the blood stream, they also noted that fasted cats appeared to set free less liver sympathin than cats which had recently been or were digesting meat. Cannon and Griffith (1922) found that, in general, stimulation of the hepatic nerves of cats fed on milk or meat led to a greater increase in the rate of the denervated heart than stimulation in cats fed mainly on fat or carbohydrate. There were, however, a number of cats which did not fit into this rule. When it became clear that the protein content of the diet had profound effects on the composition of the liver, viz., that the amount of liver cell cytoplasm varied directly with the quantity or quality of dietary protein (Addis, Lee, Lew, and Poo, 1940 ; Kosterlitz, 1947 ; Campbell and Kosterlitz, 1950, 1951), it was thought desirable to examine the possibility of a causal relationship between the results obtained by Cannon and his collaborators and the biochemical changes in the liver.

The evidence which has accumulated during the last few years has made it almost certain that the sympathin liberated after stimulation of the hepatic nerves is identical with *l*-noradrenaline (Gaddum and Goodwin, 1947 ; Mann and West, 1950 ; West, 1950). In their experiments, Cannon and Uridil (1921) and Cannon and Griffith (1922) were not in a position to match the increases in heart rate found after stimulation of the hepatic nerves with that obtained after known doses of *l*-noradrenaline. Therefore they were unable to exclude the possibility that their results were partly or wholly caused by a change in sensitivity of the denervated heart and not by a change in the quantity of sympathin or noradrenaline liberated. Evidence is presented in this paper that the variations in the increase in heart rate after stimulation of the hepatic nerves are due to such fortuitous variations in the sensitivity of the acutely denervated heart and not to effects of dietary protein.

METHODS

General design.—The amount of noradrenaline liberated after stimulation of the hepatic nerves is determined by its action on the chronically denervated nictitating membrane and on the rate of the acutely denervated heart, before and after cocaine.

It has been shown recently (Innes and Kosterlitz, 1950, 1951) that the effect of noradrenaline on the acutely denervated heart differs from that of adrenaline in that the chronotropic action of noradrenaline is usually, but not always, smaller than that of adrenaline. Furthermore, cocaine potentiates the chronotropic action of noradrenaline but not that of adrenaline. In the exceptional cases, when noradrenaline is more effective than adrenaline, the potentiating effect of cocaine is small or absent. In order to correlate the amount of noradrenaline liberated with the protein content of the diet, use is made of the fact that the protein content of a liver cell, expressed as the ratio liver protein N/deoxyribonucleic acid P (DNA-P) is a function of the quantity and quality of dietary protein (Kosterlitz, 1947; Campbell and Kosterlitz, 1950, 1951).

Operative.—In all cats in which the heart was to be denervated in the acute experiment (DH series) the right adrenal was removed and the left adrenal denervated in a preliminary operation performed under nembutal or nembutal-ether anaesthesia two to three weeks before the acute experiment. At the same time, the right nictitating membrane was postganglionically denervated by extirpation of the superior cervical ganglion. In cats in which the chronically denervated nictitating membrane was the sole indicator for liver sympathin (N series) the adrenals were removed during the acute experiment.

Experimental.—For one week before the acute experiments the cats were placed on either a low-protein diet (potatoes), the stock diet (meat, potatoes, and milk), or a high-protein diet (meat, fish, and milk).

Most of the acute experiments were performed under nembutal anaesthesia, but ether was used for a small number in order to imitate the conditions of Cannon and his collaborators as closely as possible. Both vagi were divided and the middle cervical ganglia removed. Then both stellate ganglia were excised by the antero-lateral approach through the first intercostal spaces. For the isolation of the hepatic nerves, the first step was the dissection and cannulation of the common bile duct. Then the portal vein was freed of surrounding tissue, the pancreatico-duodenal artery identified and divided together with any remaining tissue between the liver and the duodenum, and finally the hepatic nerves separated from the hepatic artery and cut as far centrally as possible.

The hepatic nerves were stimulated with shielded silver electrodes. The Ritchie-B.N.I. square wave stimulator (Walter and Ritchie, 1945) was used, and the stimulus, lasting 15 seconds, was almost always 20 V., 1 msec., 40 cyc./sec. The contractions of the nictitating membrane were recorded with an isotonic lever.

The arterial B.P. was measured in the femoral artery with a mercury manometer, heparin being used as anticoagulant. The heart rate was determined from the B.P. tracing for 30 seconds preceding the stimulation of the hepatic nerves (basal heart rate) and for 90 or 120 seconds after stimulation. The values are given in beats/min.

Stock solutions of the sympathomimetic drugs were prepared by dissolving *l*-adrenaline in 0.01 N-HCl, *dl*-noradrenaline hydrochloride and *l*-noradrenaline D-bitartrate monohydrate in water. All solutions contained 1 mg. base/ml. The final dilutions were made up with Ringer-Locke solution containing 1 mg. ascorbic acid/1 ml. The solutions containing the drugs were injected in quantities of 0.1–0.4 ml. into the femoral vein, the injection always taking 15 seconds. All values are given as μg. base.

It was usually not difficult to match the contractions of the nictitating membrane after stimulation of the hepatic nerves with those after injection of noradrenaline. This was, however, almost impossible for the chronotropic action, since the increases in heart rate could only be counted after the end of the experiment. For this reason the increase in heart rate after stimulation of the nerves was very often smaller than that after the smallest dose of noradrenaline. To give some idea of the quantity which would have matched in these instances, the ratio, 100 × increase in heart rate after stimulation of hepatic nerves to increase in heart rate after smallest dose of noradrenaline, is also

given in Table I. A similar procedure was adopted for the nictitating membrane, when necessary.

In all experiments in which the acutely denervated heart was used and in a few experiments with the chronically denervated nictitating membrane alone, the stimulations and the injection of the matching doses were repeated after the intramuscular injection of cocaine hydrochloride (8 mg./kg.).

Chemical.—At the end of the experiment samples of all liver lobes were removed, pooled, and a sample of 2 g. weighed for the estimation of deoxyribonucleic acid P (DNA-P) and protein N as described previously (Campbell and Kosterlitz, 1949, 1951).

TABLE I

THE EFFECT OF DIETARY PROTEIN ON THE AMOUNT OF *l*-NORADRENALINE LIBERATED AFTER STIMULATION OF THE HEPATIC NERVES

Exp.	Protein content of diet	Protein N/DNA-P	Approximate quantity of *l*-noradrenaline (μg.) liberated as estimated from the response of	
			Nictitating membrane	Denervated heart
DH 5	Low	76	<0.5 (70)	<0.5 (50)
N 60	Low	86	0.75–1.0	
N 42	Medium†	87	0.25–0.5	
N 69	Low	90	0.5	
DH 23	Low	91	0.6–1.1	0.6
DH 19	Low	97	<0.6 (70)	<0.6 (70)
DH 28	Low	101	<0.4 (30)	0.4
N 48	Low	105	0.5	
DH 14	Medium	105	<0.6 (70)	<0.6 (40)
N 46	Medium	106	0.13–0.25	
N 43	Medium	107	0.25	
N 62	High†	110	0.25	
N 36	Medium	113	0.5	
N 39	Medium	115	0.5	
N 52	High†	116	0.5	
N 35	—	121	1.0	
DH 16	Medium	123	0.6	0.6
DH 20	Medium	125	<0.6 (30)	<0.6 (10)
N 68	High	127	1.0	
N 55	High	128	0.25–0.5	
N 65	High	128	0.25	
DH 74*	High	134		<0.4 (60)
N 51	High	139	0.25–0.5	
N 54	High	139	0.5	
DH 81*	High	140	0.4	<0.4 (70)
DH 26	High	143	<0.4 (40)	<0.4 (70)
N 59	High	144	0.25	
DH 78*	High	144	0.4 (60)	<0.4 (60)
DH 27	High	144	0.4–0.8	0.4–0.8
DH 21	Hign	145	<0.6 (70)	<0.6 (50)
DH 22	High	154	<0.6 (40)	<0.6 (30)
DH 80*	High	158	0.8	0.4–0.8
DH 77*	High	168	0.4	<0.4 (50)
DH 67*	High	177	0.8	0.4

* In these experiments the anaesthetic was ether; in all other experiments nembutal (45 mg./kg.) was used.

† These animals were poor eaters.

The figures in parentheses are the ratios 100 × increase in heart rate after stimulation of the hepatic nerves/increase in heart rate after the smallest dose of *l*-noradrenaline. All doses are given as *l*-noradrenaline. When *dl*-noradrenaline was used for matching (in experiments with the code letter "N"), the dose of *dl*-noradrenaline was twice that given in the Table.

RESULTS

The protein content of a liver cell or rather of a unit of liver cells of the cat, expressed as the ratio protein N/DNA-P, increased with rising dietary protein intake (Table I) in the same way as was shown in the rat (Campbell and Kosterlitz, 1950). This ratio may therefore be used to indicate the amount of protein eaten.

The increase in heart rate after stimulation of the hepatic nerves varied considerably from experiment to experiment. Usually the increase was about 5 beats/min., quite independently of the protein content of the liver cells (Fig. 1). Although

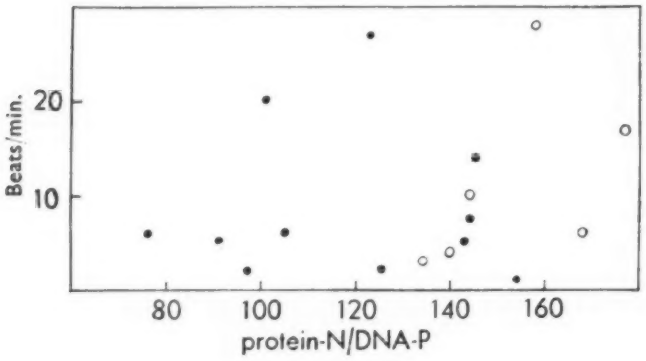

FIG. 1.—Increase in heart rate during 1½ min. after stimulation of hepatic nerves in cats with livers of varying protein content. The hepatic nerves were stimulated for 15 sec. with rectangular pulses, 20 V., 1 msec., 40 cyc./sec. The dots and circles indicate values obtained from cats under nembutal and under ether respectively.

the responses in 4 out of 16 cats (Exps. DH 16, 28, 67, 80) showed an increase of 15 or more beats/min., these were in no way correlated with high protein contents of the liver cells. Further, these responses were not due to the liberation of particularly large quantities of noradrenaline but to an increased sensitivity of the heart to amounts which usually caused increases in heart rate of rather less than 10 beats/min. The reason for the peculiar responses of these four cats is probably to be found in the fact that acute denervation alone fully sensitized the heart and thus led to a greater response than in the majority of cats. Cocaine had no potentiating effect in these cats (e.g., Fig. 2, Exp. DH 16).

There is a fair agreement between the results obtained with the nictitating membrane and those with the acutely denervated heart as indicator (Table I). It is obvious that there is no correlation between the amount of noradrenaline secreted after stimulation of the hepatic nerves and the protein content of the liver cells and therefore of the protein content of the diet. It should be noted that the cats with the highest protein N/DNA-P ratios were actively digesting fish or protein during the experiment. This is of importance, since Cannon and Griffith (1922) observed the greatest increases in heart rate in such cats.

A more detailed analysis is given of three experiments in each of which approximately 0.6–0.8 μg. of *l*-noradrenaline was secreted after stimulation of the hepatic nerves (Fig. 2). The protein content of the liver cells was relatively low in the first cat, approximately normal in the second, and high in the third. The increase in heart rate after stimulation of the hepatic nerves was small in the first and third cats but large in the second. Cocaine potentiated the small responses obtained after stimulation of the hepatic nerves and after injection of noradrenaline in cats 1 and 3, but not the large responses in cat 2. It was therefore impossible to correlate,

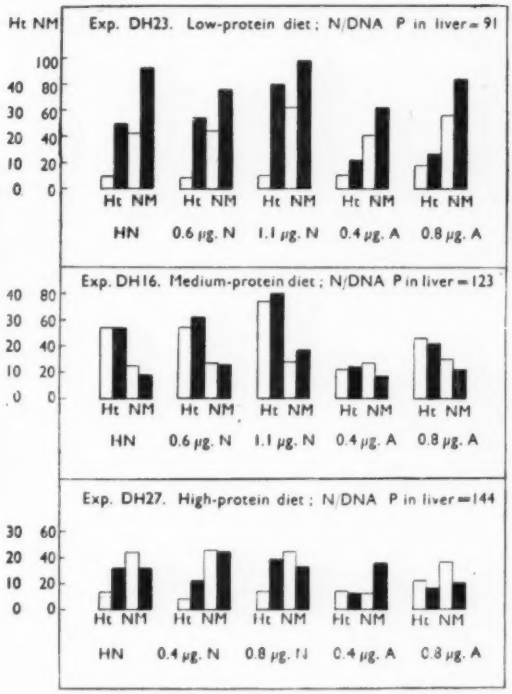
Ht NM
Exp. DH23. Low-protein diet; N/DNA P in liver = 91
100
40 80
30 60
20 40
10 20
0 0
Ht NM Ht NM Ht NM Ht NM Ht NM
HN 0.6 μg. N 1.1 μg. N 0.4 μg. A 0.8 μg. A
Exp. DH16. Medium-protein diet; N/DNA P in liver = 123
40 80
30 60
20 40
10 20
0 0
Ht NM Ht NM Ht NM Ht NM Ht NM
HN 0.6 μg. N 1.1 μg. N 0.4 μg. A 0.8 μg. A
Exp. DH27. High-protein diet; N/DNA P in liver = 144
30 60
20 40
10 20
0 0
Ht NM Ht NM Ht NM Ht NM Ht NM
HN 0.4 μg. N 0.8 μg. N 0.4 μg. A 0.8 μg. A

FIG. 2.—The effects of stimulation of the hepatic nerves and the injection of adrenaline and noradrenaline on the rate of the acutely denervated heart and on the chronically denervated nictitating membrane. The clear columns indicate effects before, and the shaded ones after, the intramuscular injection of cocaine hydrochloride (8 mg./kg.). Ht = increase in heart rate (beats/min.) during first 1½ min.; NM = maximal contraction of nictitating membrane in mm. of record. N = *l*-noradrenaline; A = *l*-adrenaline. HN = hepatic nerves stimulated.

first, the increase in heart rate with the amount of noradrenaline liberated, and, secondly, the quantity of noradrenaline secreted with the protein content of the liver cells, and thus with the amount of dietary protein ingested. As was to be expected, the responses of the denervated heart and the denervated nictitating membrane after stimulation of the hepatic nerves were matched more readily by injecting noradrenaline intravenously than by injecting adrenaline.

DISCUSSION

The experimental results reported in this paper make it impossible to uphold the views of Cannon and Uridil (1921) and Cannon and Griffith (1922) on the possible relationship between the protein content of the diet and the amount of sympathin or noradrenaline liberated after stimulation of the hepatic nerves. It would appear that Cannon and his collaborators were misled by their inability to match the chronotropic responses of the denervated heart against noradrenaline and thus exclude variations in sensitivity of the heart. Cannon and Griffith (1922) themselves stated that a number of cats did not fit into their hypothesis, cats which had been fed on a high-protein diet but did not show a marked increase in heart rate after stimulation of the hepatic nerves. These exceptions gain additional significance in the light of the results reported in this paper.

These conclusions have come as a disappointment to the present authors, who had hoped that the findings of Cannon and his collaborators might form the basis of an interpretation of the physiological significance of the changes occurring in the composition of the liver cell when the protein content of the diet is varied. Perhaps

Cannon himself doubted the validity of the earlier experiments when he and Lissák (1939) discussed the significance of the fact that the parenchymal cells of the liver did not receive sympathetic fibres.

Summary

1. The findings of Cannon and Uridil (1921) and Cannon and Griffith (1922), viz., that cats fed on a diet rich in protein liberate more sympathin on stimulation of the hepatic nerves than cats fed on diets poor in protein, have been re-examined.

2. The responses of the acutely denervated heart and of the chronically denervated nictitating membrane to stimulation of the hepatic nerves were matched with noradrenaline injected into the femoral vein. No correlation was found between the amounts of noradrenaline secreted and the amount of dietary protein eaten.

Grants by the Medical Research Council (to H. W. K.) are gratefully acknowledged. We wish to thank Dr. M. L. Tainter, of the Sterling-Winthrop Research Institute, for a generous gift of *dl*-noradrenaline. The sample of *l*-noradrenaline D-bitartrate monohydrate, which was made available by the Sterling-Winthrop Research Institute, was obtained through the courtesy of Bayer Products, Ltd.

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NORADRENALINE AND THE SUPRARENAL MEDULLA

BY

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Noradrenaline is widely distributed in the body and has been found in all nerves containing adrenergic fibres. The amount of adrenaline occurring together with noradrenaline in such nerves is usually only a few per cent of the total catechol amines, showing that noradrenaline is the more important adrenergic mediator.

In extracts of suprarenal glands, however, the relative amounts of adrenaline and noradrenaline vary greatly in different animals. In the ox and pig, 30–40 per cent of the total amines is noradrenaline (Holtz and Schümann, 1949). It has been isolated in pure crystalline form from the adrenals of cattle (Bergström, Euler, and Hamberg, 1949), and has been shown (Holtz and Schümann, 1950) to be present in the adrenal medulla of the dog, cat, and man. Very little has been found in rabbit suprarenals (West, 1950b).

Evidence that noradrenaline is a precursor of adrenaline in the suprarenal medulla has been obtained by several workers. Firstly, Bülbring (1949) showed that minced suprarenal tissue from dogs and cats, when incubated with adenosine triphosphate and choline, converts noradrenaline to adrenaline. Secondly, Bülbring and Burn (1949b) observed a disappearance of noradrenaline and a corresponding increase in the amount of adrenaline in the perfused suprarenal gland of the dog. The proportion of noradrenaline and adrenaline which is released from the gland by nerve stimulation in cats (Bülbring and Burn, 1949a ; West, 1950a) probably depends on the relative amounts present, and there is no convincing evidence that one amine is released alone. Lastly, when the suprarenal medulla of the rat is depleted by the injection of insulin (Burn, Hutcheon, and Parker, 1950b ; Outschoorn, 1951), both adrenaline and noradrenaline are lost from the gland, though it is more depleted of the former amine. When insulin is given to rabbits (West, 1951) similar results are observed. These observations suggest that, when the suprarenal glands are exhausted, mostly adrenaline is poured forth into the blood stream and relatively more noradrenaline is found in the gland.

The proportion of noradrenaline in extracts of tumours of the adrenal medulla of man (Holton, 1949) is higher than that found in normal glands (Shepherd and West, 1951). This difference may be related to the fact that the tumour cells are relatively undifferentiated and thus may be more comparable with embryonic tissue than with adult tissue. Hence the present investigation was undertaken to discover what precursors of adrenaline are present in the suprarenal medulla of young animals, including that of man. If such precursors, or their relative amounts, differed from those found in the adult state, it might be possible by taking animals at different

ages to correlate the change-over with a certain stage in development. In addition, another method might be provided of studying adrenaline formation in the body.

METHODS

Preparation of the adrenal extracts.—The human glands were secured from the post-mortem room as soon as possible after death ; occasionally the delay was as long as 48 hours, but the majority of specimens were obtained before 24 hours had elapsed. After the capsule and the fatty tissue adhering to it had been removed, the glands were weighed and ground with sand and 0.1–1 ml. of 0.01 N-HCl/g. The acid extracts were centrifuged, and the clear supernatants were assayed for their noradrenaline and adrenaline contents as described below. In many adult specimens, the cortices were separated from the medullae. Each part was then weighed, extracted, and assayed separately.

The adrenal glands of rabbits, cats, guinea-pigs, dogs, and domestic fowl were secured after killing the animals by a blow on the head or by an overdose of urethane. The glands were dissected free of capsules, weighed, and ground in a mortar with sand and 1–5 ml. 0.01 N-HCl/g. After centrifuging, the extracts were assayed. In a few experiments with guinea-pig glands, a similar volume of 10 per cent trichloroacetic acid was used instead of hydrochloric acid for the preparation of the extracts. In these experiments, excess trichloroacetic acid was removed with ether before the solution was assayed.

Assay methods.—All extracts were assayed for their adrenaline and noradrenaline contents by paper chromatography and by the action on the blood pressure and normal nictitating membrane of a spinal cat (Burn, Hutcheon, and Parker, 1950a). In certain experiments with guinea-pig extracts, colorimetric assays (Euler and Hamberg, 1949b) were also carried out. The method is discussed in detail under the results of guinea-pig gland assays.

All chromatograms were carried out by the ascending method in a glass tank (15 by 7 by 22 in.) containing the solvent to a depth of $\frac{1}{2}$ in. The solvent was prepared by shaking *n*-butyl alcohol (4 vol.), glacial acetic acid (1 vol.), and water (5 vol.) together and discarding the lower layer. The butyl alcohol and acetic acid were of ordinary reagent quality and were not purified before use. Sheets of Whatman No. 4 filter-paper, 12 in. wide, were suspended from horizontal glass rods placed 18 in. above the liquid surface, the paper being kept taut by means of a thin glass tube 12 in. long, closed at both ends and threaded through four vertical slits $\frac{1}{8}$ in. long and 3 in. apart cut near the bottom of the sheet. The top of the tank was sealed by a vaselined glass plate.

Test solutions were applied from a graduated glass syringe as single or replicate drops each 0.01 ml., 1 in. apart, along a line 2 in. from the foot of the paper and at least 2 in. from the lateral edges of the paper where the flow tended to be erratic. Usually 0.02 ml. was sufficient, although for very weak extracts 0.08 ml. was necessary. When the drops had dried, chromatography was carried out at room temperature for 18 hours, during which time the solvent travelled 12 in. from the starting line. After drying for 15 minutes in a cabinet through which a current of warm air circulated, the paper was sprayed with a 1 per cent (w/v) aqueous solution of potassium iodate. On development in an air oven at 100–110° C. for not more than two minutes, adrenaline and noradrenaline were rendered visible as pink and violet spots respectively. Care was taken to avoid overheating, which causes both spots to become brown and indistinguishable in colour. At room temperature, the spots assume a uniform brown colour within a few hours. Since the colours are viewed against a white background, the method is more sensitive than the original ferricyanide method (James, 1948), in which the spots are viewed against a yellow background. The use of potassium iodate as a developer for adrenaline and noradrenaline on chromatograms has recently been described by Burn, Langemann, and Parker (1951), who employed a 10 per cent solution of the salt

in 5 per cent acetic acid. In our experience this solution showed a marked tendency to crystallize in the atomizer during spraying, gave a less striking colour difference between adrenaline and noradrenaline, and would not detect such small quantities as the aqueous solution used by us.

Estimates of adrenaline and noradrenaline contents of adrenal extracts were obtained by comparison of the intensities of the spots with those from standard solutions of the two amines, and values agreed well with those obtained by parallel biological and colorimetric assay. By the iodate method described above, spots containing 1 μg. adrenaline or 2 μg. noradrenaline can be readily detected. In no experiment was dihydroxyphenylalanine or hydroxytyramine detected in adrenal extracts by the chromatographic method. The R_f values and colours of the spots given by these substances as well as by adrenaline and noradrenaline are recorded in Table I.

TABLE I

R_f VALUES OF FOUR AMINES IN BUTANOL-ACETIC ACID-WATER SOLVENT

Amine	Rf value	Colour of spot on development with 1% KIO_3
Dihydroxyphenylalanine	0.19	Greyish-violet
Noradrenaline	0.28	Violet
Adrenaline	0.36	Pink
Hydroxytyramine	0.39	Orange-brown

Solutions of *l*-adrenaline, *l*-noradrenaline bitartrate, hydroxytyramine hydrochloride, and *dl*-dihydroxyphenylalanine in 0.01 N-HCl were used throughout this study.

RESULTS

Human adrenal glands.—The mean values of extracts from 31 adult glands were 209 μg. adrenaline and 31 μg. noradrenaline/g. Hence 13 per cent of the total catechol activity exists as noradrenaline. In general, lower values for total activity were obtained in cases where the cause of death was of an infectious nature or dying prolonged. Higher figures were found in extracts where rapid deaths had occurred. So far we have been unable to obtain for comparison the glands of healthy individuals who died a sudden or violent death. It must not be assumed, therefore, that the mean values quoted above represent the normal amounts found in the healthy gland.

In 10 of the samples, the activities of the medullary and cortical components have been compared. The mean values were 1,260 μg. adrenaline and 214 μg. noradrenaline/g. fresh medulla, and 109 μg. adrenaline and 3 μg. noradrenaline/g. fresh cortex. Hence 15 per cent of the total in the medulla is noradrenaline, whereas in the cortex it is only 3 per cent. This suggests that noradrenaline is formed in the medulla, although no proof has yet been obtained that it is actually produced in the gland.

In a series of 25 children under 70 days, the mean values were 2 μg. adrenaline and 30 μg. noradrenaline/g. fresh tissue. The position is therefore reversed to that seen in adults (Fig. 1). Over 90 per cent of the total catechol amines in human embryonic tissue is noradrenaline, a result similar to that seen in adult suprarenal

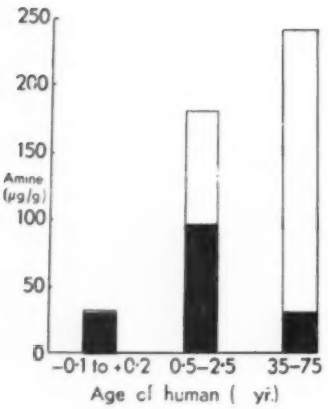

FIG. 1.—The influence of age on the amine content (μg./g. fresh tissue) of suprarenal glands of man. Shaded area is noradrenaline; plain area is adrenaline.

medullary tumours. The glands of three children aged 180, 210, and 912 days were extracted and assayed, and the relative noradrenaline values were 67, 50, and 32 per cent respectively.

Cat adrenal glands.—The mean values for extracts of glands of 12 adult cats were 601 μg. adrenaline and 369 μg. noradrenaline/g. Therefore some 38 per cent of the total activity is noradrenaline. These values agree with those reported earlier by Bülbring (1949) and Euler (1950). When animals between 2 and 3 days old were used, the noradrenaline proportion in the extracts was found to be significantly higher, representing about 82 per cent of the total amines. The mean values for extracts of 20 embryonic glands were 141 μg. adrenaline and 642 μg. noradrenaline/g. The glands of three kittens *in utero* were extracted and found to contain 150 μg. adrenaline and 375 μg. noradrenaline/g. Over 70 per cent of the total amine content of these glands therefore exists as noradrenaline (Fig. 2).

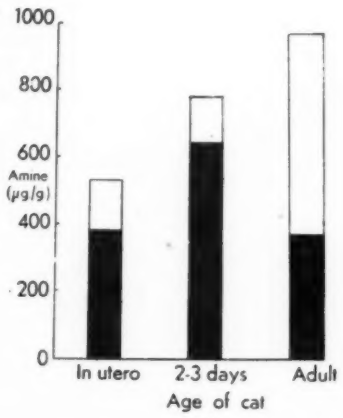

FIG. 2.—The influence of age on the amine content (μg./g. fresh tissue) of suprarenal glands of cat. Shaded area is noradrenaline; plain area is adrenaline.

Rabbit adrenal glands.—The mean values for extracts of glands of 56 adult rabbits were 470 μg. adrenaline and 10 μg. noradrenaline/g. Therefore only 2 per cent of the total activity is noradrenaline. In very young rabbits (secured *in utero*), total activity in the glands was reduced and was composed entirely of noradrenaline.

By the third day of life, total activity had increased four- or five-fold, and large amounts of adrenaline were now present. By using a large series of animals, it was possible to trace the change-over in the gland from almost entirely pure noradrenaline to a high percentage of adrenaline (Table II and Fig. 3).

TABLE II

THE INFLUENCE OF AGE ON THE NORADRENALINE AND ADRENALINE CONTENTS OF THE SUPRARENAL GLANDS OF RABBITS

No. of expts.	Age	Weight (g.)	Activity of gland extract (μg./g.)			% Noradren. in total
			Adren.	Noradren.	Total	
12	*In utero*	45	0	200	200	100
4	1 hour	40	250	250	500	50
23	6 hours	50	181	544	725	75
3	1 day	60	75	670	745	90
3	2 days	50	297	603	900	67
10	2 days	55	574	437	1,011	43
4	8 days	105	667	125	792	16
56	Adult	2,000	470	10	480	2

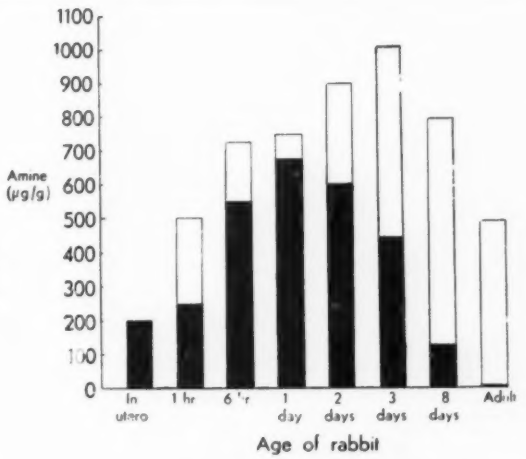

FIG. 3.—The influence of age on the amine content (μg./g. fresh tissue) of suprarenal glands of rabbit. Shaded area is noradrenaline; plain area is adrenaline.

Guinea-pig adrenal glands.—The guinea-pig is an interesting animal, for it possesses relatively the largest adrenal of any animal. Euler, Hamberg, and Purkhold (1949) and Schuler and Heinrich (1949) investigated the guinea-pig suprarenals and estimated by chemical assay that there are 338–400 μg. catechols per g. fresh tissue. Of this total, over 22 per cent has been reported as noradrenaline. Our early results on adult glands indicated that total catechols were about 132 μg./g. and that the proportion of noradrenaline seldom exceeded a few per cent of the total. Noradrenaline was in fact present in only 12 out of 41 experiments. Later determinations on animals secured from another source (Sussex) confirmed this finding, and for 60 animals no more than 3 per cent of the total catechols (mean value 127 μg./g.) was noradrenaline. Since results on total activity and relative noradrenaline content differed widely from those previously reported, further work was carried out to determine the possible sources of error.

We extracted the glands of four adult guinea-pigs with 10 per cent trichloroacetic acid, and, after removal of the excess acid with ether, we subjected the extracts to biological and chemical assay. The chemical assay was that used by Euler, Hamberg, and Purkhold (1949), and depends on the development of colour with iodine. Iodoadrenochrome is formed in 1½ minutes at *p*H 4, whereas only 10 per cent of iodonoradrenochrome is formed at this *p*H. On three-minute treatment with iodine at *p*H 6, maximal formation of both quinones is attained. The difference in oxidation rates at these two *p*H values is the basis of this method of estimating the two substances separately (Euler and Hamberg, 1949a). The results obtained on the four extracts are shown in Table III. Two important conclusions are indicated: (*a*) the total amine content estimated by the iodine method was about twice to four times

TABLE III

ESTIMATIONS OF CATECHOLS (μg./g.) IN TRICHLOROACETIC ACID EXTRACTS OF ADRENAL GLANDS OF ADULT GUINEA-PIGS

Extract	Chemical assay				Biological assay			
	Adren.	Noradren.	Total	% Noradren.	Adren.	Noradren.	Total	% Noradren.
1	200	38	238	16	150	0	150	0
2	150	24	174	14	120	2	122	2
3	240	88	328	27	80	0	80	0
4	225	125	350	35	125	0	125	0

that found by biological determination, and (*b*) the proportion of noradrenaline in the mixture was much higher, values being about 10 times those found biologically. It was then found that the presence of precursors of adrenaline may produce erroneous results by this method. Dihydroxyphenylalanine, for example, gives a quinone at *p*H 4, although it is maximal at *p*H 6. Hydroxytyramine gives no colour with iodine at *p*H 4, but is maximal at *p*H 6. Both these substances may be present in adrenal extracts and may affect the iodine reaction, without interfering greatly with the biological assay. A false noradrenaline proportion and raised total activity may result. In addition, Euler (1950) has suggested that extraction of organs with trichloroacetic acid may give a higher yield of active material.

We have carefully examined the extracts of guinea-pig glands by paper chromatography and have never found any hydroxytyramine or dihydroxyphenylalanine. We conclude, therefore, that extraction with trichloroacetic acid and estimation by the iodine reaction yields erroneous results with guinea-pig gland extracts. It is possible that the large cortex seen in the guinea-pig contains a material which enhances the iodine reaction. There may also be a real distinction in the catechol content of the adrenal glands between different breeds. Elliott and Tuckett (1906) have already reported a difference in weights of adrenal glands of English and French guinea-pigs.

When we examined the extracts of glands removed from young guinea-pigs, values for total activity and relative noradrenaline content were much higher than those found in adults. Embryonic tissue of the guinea-pig therefore contains predominantly noradrenaline (Table IV and Fig. 4).

Dog adrenal glands.—Previous workers (Bülbring, 1949; Bülbring and Burn, 1949b) obtained evidence that noradrenaline was present with adrenaline in the

TABLE IV

THE INFLUENCE OF AGE ON THE NORADRENALINE AND ADRENALINE CONTENTS OF THE SUPRARENAL GLANDS OF GUINEA-PIGS

No. of exp's.	Age	Weight (g.)	Activity of gland extract (μg./g.)			% Noradren. in total
			Adren.	Noradren.	Total	
5	*In utero*	10	0	50	50	100
3	At term	33	250	125	375	33
1	1 day	40	75	150	225	66
2	2 days	45	75	150	225	66
1	7 days	60	50	250	300	83
1	18 days	135	250	100	350	40
1	20 days	150	250	50	300	17
1	23 days	167	250	0	250	0
2	26 days	175	185	0	185	0
1	31 days	250	125	0	125	0
60	Adult	500	124	3	127	3

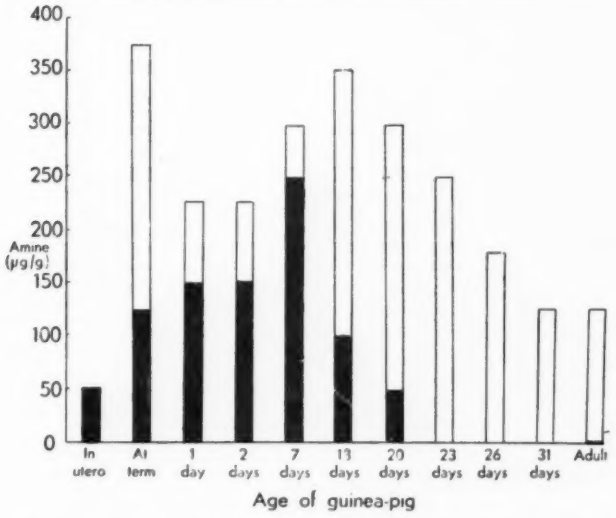

FIG. 4.—The influence of age on the amine content (μg./g. fresh tissue) of suprarenal glands of guinea-pig. Shaded area is noradrenaline; plain area is adrenaline.

suprarenal glands of the dog, but the amount varied considerably (from 0 to 52 per cent of the total amines). We investigated the adrenal glands of puppies to see if their embryonic tissue produces predominantly noradrenaline. Results shown in Fig. 5 indicate that there is not such a marked difference in this species between the relative amine contents of glands of young and old animals, as is seen in guinea-pigs, rabbits, and cats. Perhaps dogs younger than 6 days may provide the necessary data.

Adrenal glands of domestic fowl.—Whereas the adrenal glands of the guinea-pig, rabbit, and cat increase in size as the body grows, those of the fowl do not increase in weight after the third month of life (Elliott and Tuckett, 1906). In addition, the medulla forms a very large fraction of the whole gland, and there is little change with age in the ratio of cortical size to medullary size. The results we have obtained using the adrenal glands of fowls of all ages are shown in Table V. Noradrenaline predominates in the gland even in the adult state, and it is interesting to note that there is little change with age in the relative noradrenaline content.

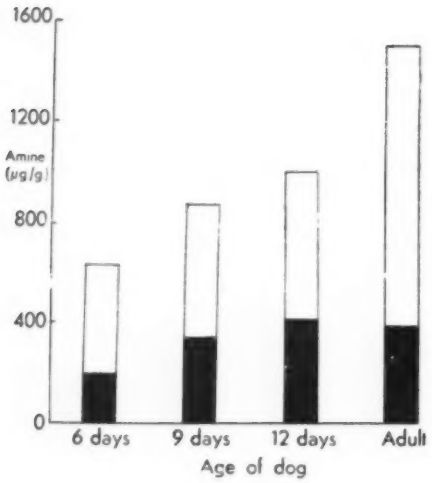

FIG. 5.—The influence of age on the amine content (µg./g. fresh tissue) of suprarenal glands of dog. Shaded area is noradrenaline; plain area is adrenaline.

TABLE V

THE INFLUENCE OF AGE ON THE NORADRENALINE AND ADRENALINE CONTENTS OF THE SUPRARENAL GLANDS OF DOMESTIC FOWL

No. of expts.	Age (days)	Weight (g.)	Activity of gland extract (µg./g.)			% Noradren. in total
			Adren.	Noradren.	Total	
25	1	40	3,300	4,950	8,250	60
10	7	70	3,325	6,175	9,500	65
2	77	650	4,000	6,000	10,000	60
17	110	1,000	2,020	8,080	10,100	80
13	200	2,000	3,375	7,875	11,250	70

DISCUSSION

The results indicate that embryonic tissue in suprarenal glands of man, cat, rabbit, guinea-pig, and dog contains a high proportion of noradrenaline and a very small amount of adrenaline, a finding which is also characteristic of human medullary tumours. While this work was in progress, Holton (1951) obtained a similar result when she compared the adrenal glands of calves with those of bullocks. Although we have been unable to obtain any evidence of the presence of hydroxytyramine and dihydroxyphenylalanine in embryonic gland extracts, large amounts of noradrenaline show that this amine must be a precursor of adrenaline in these mammals. In fact, noradrenaline itself may be the hormone of the gland in the early days of life.

The exact area in the adrenal gland of the adult where methylation of noradrenaline occurs is still an open question. The medullary component of the adult human gland is about one-tenth of the total, so that in the glands where the cortices and medullae were divided and extracted separately it is possible to calculate the amounts of each amine present in each component. Thus, much more noradrenaline is present in the medulla ($214/10=21$ µg./g. gland) than in the cortex ($3 \times 0.9=2.7$ µg./g. gland), although the adrenaline concentrations are of the same order (126

μg./g. in the medulla and 91 μg./g. in the cortex). We have noted that, as the cortical size relative to that of the medulla increases, so the relative amount of noradrenaline in the adult gland decreases ; even within the same species, this finding is true (Table VI). When there is little change with age in the ratio of cortical size to medullary size (as in the fowl, Table V), there is also little change in the relative amount of noradrenaline in the gland. The ratio of cortical size to medullary size is therefore related to the proportion of noradrenaline found in the adrenal gland.

TABLE VI

THE RELATIONSHIP BETWEEN THE MEDULLA-CORTEX RATIO AND THE RELATIVE NORADRENALINE CONTENT OF ADRENAL EXTRACTS OF ANIMALS

Animal	Medulla : Cortex	% Noradrenaline in total amines
Fowl	1 : 1	80
Whale	1 : 2	80*
Pigeon	1 : 2	55
Dog	1 : 5	27
Man	1 : 9	13
Cat	1 : 18	38
Rat	1 : 20	9
Rabbit	1 : 40	2
Guinea-pig	1 : 63	3
Young guinea-pig (7 days)	1 : 6	83

* Burn, Langemann, and Parker (1951).

It has been found that the adrenal cortex of the guinea-pig contains a material which may enhance the iodine reaction of Euler and Hamberg (1949) and so produce erroneous results both for total activity and for the proportion of each amine present. When the cortical extract of some human glands was tested colorimetrically (iodine method), biologically, and by paper chromatography, the results by the last two methods were in agreement, but the chemical method gave an answer which was about twice this amount. On the other hand, medullary extracts of the same glands gave comparable results by all three methods. In addition, an erroneous result (three times that found biologically) was obtained when the iodine reaction was applied to some extracts of rabbit glands where a large cortical component is present, but, when applied to extracts of cat and whole human glands, good agreement was reached by all tests. All these findings suggest that there is a cortical material which may upset colorimetric estimations of total amine activity and of proportions of each present.

It is of course accepted that the human glands obtained at death are in an exhausted state, and hence values found must of necessity be low. To obtain a rough estimate of the true value, we first examined the gland extracts of four adult cats which had been dead for more than 20 hours. Despite the fact that the noradrenaline percentage for this animal was within the normal adult range, total activity had dropped to 204 μg./g., i.e., about one-quarter of the normal value. Secondly, in two guinea-pigs which apparently were not feeding well, and which died after being very subdued for two days, we found that the total amine content of

the adrenal gland was reduced to 25 μg. adrenaline/g., a value about one-fifth of that normally found. Lastly, in 11 experimental rabbits subjected to ether anaesthesia for long periods, the mean value for total activity was 110 μg. adrenaline/g. This is about one-quarter of that found in glands of freshly killed rabbits. Hence we conclude that the values we have obtained on human adrenal glands are significantly less than those present in the normal unexhausted gland.

Conclusion

1. The sympathomimetic amine present in embryonic adrenal glands of cat, rabbit, guinea-pig, dog, and man is noradrenaline: very small amounts of adrenaline may be found.

2. In the adult glands of these mammals, it is suggested that the degree of methylation of noradrenaline is related to the relative cortical size. In animals where the cortex is large relative to the medulla (e.g., the rabbit and the guinea-pig), methylation of noradrenaline is almost complete, and often only adrenaline is found in gland extracts.

3. The total activity in exhausted adrenal glands of man is about 0.24 mg./g. (31 estimations). The results of animal experiments indicate that a total activity of about 1 mg./g. might be found in the healthy individual.

4. In no experiments were dihydroxyphenylalanine or hydroxytyramine detected in adrenal extracts by the chromatographic method.

We are grateful to Professor R. B. Hunter for his helpful advice and criticism during the course of this study. We record our thanks to Professor Lendrum, Dr. Prain, and other members of the Pathology Department of the University for their co-operation in securing human material. Mr. W. Cooper rendered valuable technical assistance.

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THE DEPRESSOR ACTION OF THE VERATRUM ALKALOIDS

BY

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In two recent papers Stutzman and his colleagues have raised some new points about the mode of action of the veratrum alkaloids upon the cardiovascular system. Using "Veriloid" ("a purified reproducible extract of *Veratrum viride* prepared by the Riker Laboratories of Los Angeles") they observed that cutting both cervical vagi eliminated the bradycardia, but did not alter quantitatively the fall of blood pressure when this extract was injected intravenously into dogs (Stutzman, Simon, and Maison, 1951). Yet there is ample evidence that cutting the vagi abolishes or very greatly reduces the fall of blood pressure and heart rate which follows the rapid intravenous injection of a single dose of crude extracts of *Veratrum album* or *Veratrum viride*, or of the pure substances veratridine, protoveratrine, or cevadine. Now, veriloid, according to Stutzman, Maison, and Kusserow (1949), is a mixture of alkaloids from which none of the previously described potent alkaloids of *Veratrum viride* has been obtained. It therefore seemed possible that veriloid contained alkaloids which acted in a different manner from those previously investigated. In the first part of this paper, by applying the methods of investigation previously used on other veratrum alkaloids, we have shown that veriloid possesses qualitatively similar properties.

Stutzman, Simon, and Maison (1951) also observed that *infusions* of germitrine, protoveratrine, germidine, germerine, and veratridine into dogs anaesthetized with pentobarbitone (nembutal) caused a fall of blood pressure which was not altered quantitatively by cutting the vagi. They therefore concluded that under these circumstances the Bezold reflex, the afferent fibres of which run in the vagi, is not responsible for the depressor action. In this respect also their results differed from those of previous workers in this field, who had used rapid single injections to test the reaction of the animal. The question is relevant to clinical practice, since a slow intravenous infusion might be expected to mimic the conditions in the circulation after a dose had been taken by mouth. Experience with the use of the veratrum alkaloids for studying the Bezold reflex over a number of years by no means agrees with the conclusions of Stutzman and his colleagues. In the first place it is not suggested that the Bezold reflex is alone responsible for the fall of blood pressure and heart rate on intravenous injection of *veratridine* in the dog, since in 1943 Krayer, Wood, and Montes showed that there was a central action, which caused a fall of heart rate. Yet the dose required to produce this central action was somewhat larger than that required to produce the Bezold reflex on injection into the

thoracic visceral circulation. In cats (Dawes, 1947) there is an even greater difference between the minimal effective dose for the Bezold reflex and that needed for a central action ; thus a large fall of blood pressure and heart rate was obtained on rapid intravenous injection of a dose, which was ineffective on injection into the ascending aorta. Some of the records published by Jarisch and Richter (1939), in which multiple small doses of veratrine had been used in the cat to produce a prolonged fall of blood pressure, which was abolished by cooling the vagi, also led the authors to conclude that the Bezold reflex was the principal agent in producing the hypotension.

These considerations have led to a brief investigation of the mode of action of *infusions* of veratridine, described in the second part of this paper.

Methods

Cats and dogs were anaesthetized with chloralose (50–70 mg./kg.). Blood pressure was recorded with a mercury manometer from the carotid artery, and injections or infusions were made into the external jugular vein. The vagi were cooled by laying them on hollow thermodes, 1 cm. long, through which cold alcohol was circulated from an ice-salt mixture reservoir ; the temperature of the thermodes was recorded by thermocouples. Heart rate was recorded by the method described by Dawes (1951). Coronary perfusions in the dog were carried out by the method described by Dawes (1947). Respiration was recorded by measuring the changes in volume of the body below the neck (Dawes, Mott, and Widdicombe, 1951). Action potentials were recorded from slips dissected from the cervical vagi, a conventional capacitance-coupled amplifier being used.

Results

Mode of action of veriloid

A more detailed study of the mode of action of veratridine (Dawes, Mott, and Widdicombe, 1951) has shown that the Bezold reflex in the cat is blocked by cooling the vagi to 9–11° C., at which temperature most vagal cardiac efferents are still active. Single injections of veriloid were therefore made in cats, and the cervical vagi were cooled during some of these injections. Cooling to 8° C. always abolished the fall of blood pressure and heart rate (Fig. 1). Cutting the vagi also abolished it, except in one cat, in which the response was greatly reduced. These observations are quite consistent with an action on the receptors for the Bezold reflex in the coronary circulation, and the one instance in which a small response remained after cutting the vagi can be attributed to a much smaller action on the central nervous system or perhaps the carotid body.

In two dogs veriloid was injected into the left circumflex coronary artery, perfused from the left internal mammary artery (cf. Dawes, 1947). As little as 0.4 μg. veriloid caused a fall of 20 mm. Hg of blood pressure, and 1 μg. caused a large hypotension and bradycardia on injection into the coronary artery ; this response was abolished on cutting the vagi (Fig. 2). Injections of the same doses of veriloid intravenously or into the right ventricle were without effect. These observations substantiate the view that veriloid does not differ from the other active veratrum alkaloids in its ability to elicit the Bezold reflex.

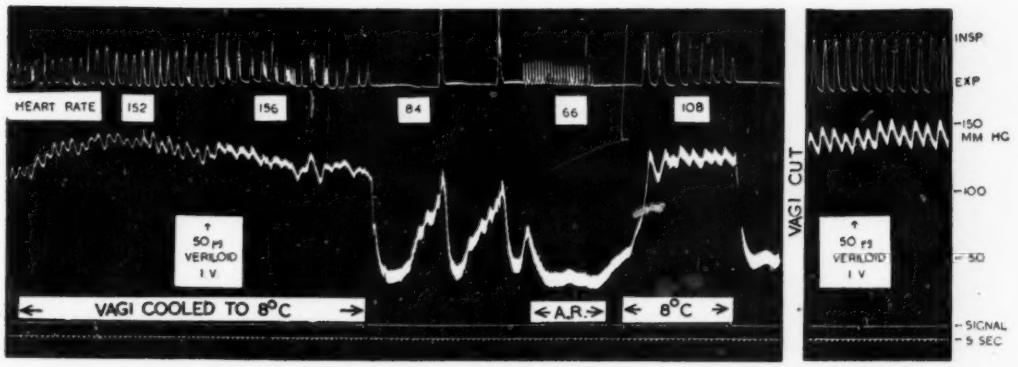

FIG. 1.—Cat, 2.8 kg., chloralose. Records of respiratory movements (above) and blood pressure (below). Both vagi were cooled to 8° C., and at 12.15 p.m. 50 µg. veriloid were injected intravenously. Two and a half minutes later the vagi were warmed, the blood pressure and heart rate fell precipitously, and breathing ceased. Artificial respiration (A.R.) was applied for a short time, and then the vagi were cooled again; the blood pressure and heart rate rose and respiration began again. After cutting the vagi the injection of the same dose of veriloid at 2 p.m. was without effect.

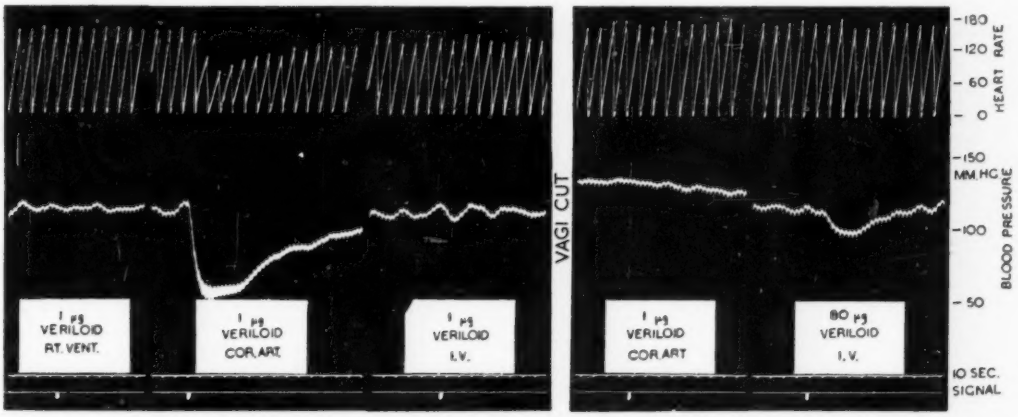

FIG. 2.—Dog, 8.0 kg., chloralose. Records of blood pressure (below) and mean heart rate (above, integrated over 10 secs.). Injection of 1 µg. veriloid into the left circumflex coronary artery (Cor. Art.) caused a fall of blood pressure and heart rate; the same dose injected into the right ventricular cavity (Rt. Vent.) or intravenously (I.V.) had no action. Cutting the vagi abolished the effect of a coronary injection, though 80 µg. injected intravenously then caused a very small fall of blood pressure.

Veriloid in both cats and dogs had, for a veratrum alkaloid, an unusually long duration of action on intravenous injection. This feature was particularly noticeable in its effect on breathing. Stutzman *et al.* (1949) observed that like other veratrum alkaloids it caused slowing or stoppage of respiration, an effect which was abolished by vagotomy, and was also abolished by cooling the vagi to 8° C. (Fig. 1). The action of veratridine in sensitizing or exciting pulmonary stretch receptors has already been described (Dawes, Mott, and Widdicombe, 1951), and it therefore seemed likely that veriloid would possess the same property, since the pulmonary stretch fibres are blocked by cooling the vagi to about 10° C. Action potentials

were recorded from single fibre preparations dissected from the cervical vagi, with a rhythm characteristic of the discharge from the pulmonary stretch receptors. Injections of veriloid greatly increased the discharge in response to normal or artificial respiratory movements, and the discharge continued at a considerable frequency even during expiration. As Fig. 3 shows, the sensitization was still present more than

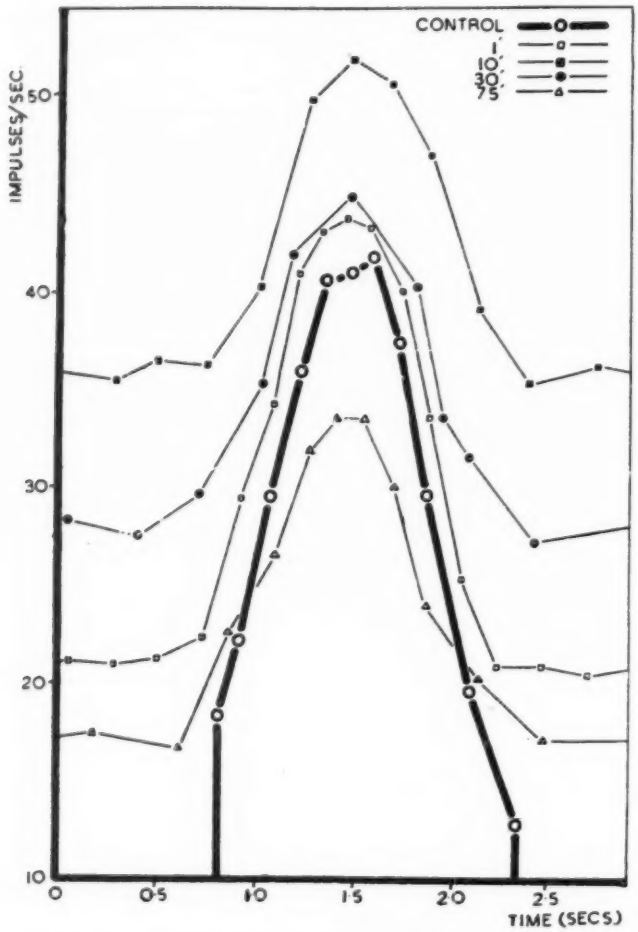

FIG. 3.—Cat, 2.8 kg., chloralose. Artificial respiration from a pump. The graphs show the discharge frequency of a single vagal pulmonary stretch fibre plotted against the artificial respiration cycle (each of which lasts 3 seconds), before and at various times after a single injection of 40 μg. veriloid. The discharge frequency is much increased after veriloid and the fibre fires continuously during expiration; the latter phenomenon lasts for more than an hour.

an hour after a single intravenous injection of 40 μg. veriloid into a cat. This is a forceful illustration of the ability of veriloid to cause repetitive discharges in afferent nerve structures, a property which seems fundamental to the characteristic activity of the veratrum alkaloids.

So far as these experiments go there is no reason to suppose that veriloid differs qualitatively in its mode of action from the pure veratrum alkaloids.

Infusion of veratridine

The second point raised by the work of Stutzman and his colleagues was the relative importance of the central and peripheral actions of the veratrum alkaloids on intravenous infusion. We infused veratridine at the rate of 2 μg./kg./min. into three cats, and during the infusion cooled the vagi to 8° C. Each infusion was continued for 10 minutes, with an hour's interval between infusions. In all three animals the results were striking and quite unequivocal. Cooling the vagi abolished the fall of blood pressure and heart rate (Fig. 4). In one cat we subsequently cut

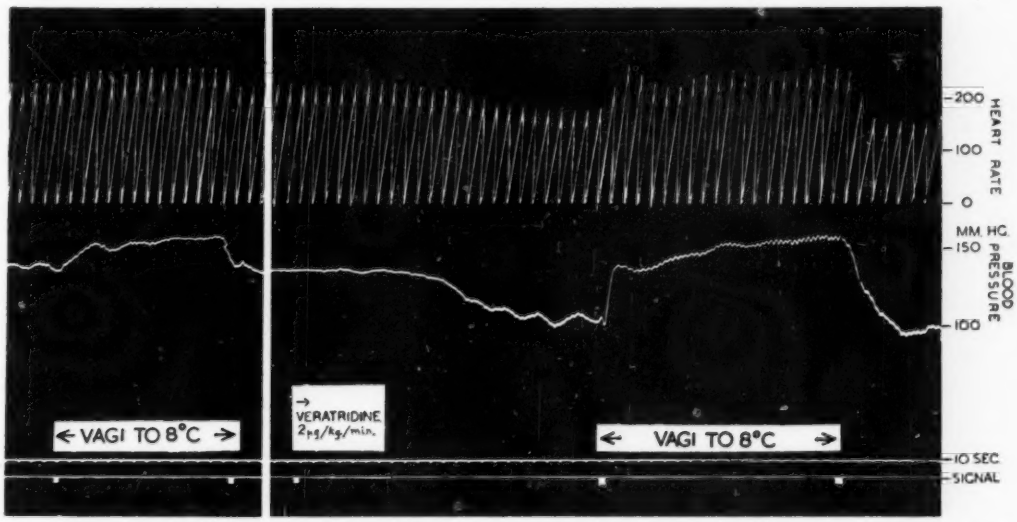

FIG. 4.—Cat, 2.9 kg., chloralose. Records of mean heart rate (above, integrated over 10 sec.) and blood pressure (below). Cooling the cervical vagi to 8° C. causes a small rise of blood pressure and heart rate. An infusion of veratridine at a rate of 2 μg./kg./min. causes a fall of blood pressure and heart rate which is abolished by cooling the vagi to 8° C.

the vagi and infused veratridine at a rate of up to 7 μg./kg./min. without any action whatsoever on blood pressure or heart rate.

In dogs our results were different and agreed more closely with those of Stutzman, Simon, and Maison (1951). In a total of 10 dogs infusions of veratridine at a rate of 1–4 μg./kg./min. gave very variable results. There was a great difference in sensitivity, but in all experiments where there was a fall of blood pressure this was accompanied by a fall of heart rate of proportionate magnitude. In two of these animals cooling the vagi to 8° C. abolished or much reduced the fall of blood pressure and heart rate. In three other dogs cooling the vagi did not significantly affect the response. In the remainder the findings were equivocal. It should be mentioned at this point that the rise of blood pressure and heart rate on cooling the vagi during an infusion of veratridine was always compared with the rise on cooling seen before and some considerable time after an infusion. The variation in these control responses explains the presence of findings which we regard as equivocal.

During these experiments both with veriloid and veratridine we encountered the phenomenon of tachyphylaxis on a number of occasions.

Discussion

The experiments with veriloid show that this mixture of alkaloids has the same general properties in its action upon the cardiovascular and respiratory mechanisms as the other potent extracts and alkaloids of veratrum.

Stutzman and his colleagues have introduced in their two papers a number of hypotheses about the mode of action of these substances. The most important of these from the clinical standpoint is their view that the Bezold reflex is in no way responsible for the fall of blood pressure during the slow infusion of a veratrum alkaloid ; yet it may cause bradycardia if the injection is rapid, since they also suppose that the *central* action of a veratrum infusion does not include bradycardia. On this basis it is not clear how they account, to take one example, for the fall of heart rate demonstrated by Kauntze and Trounce (1951) on intravenous infusion of veriloid in man. It has in fact been shown without doubt that the Bezold reflex causes both peripheral vasodilatation and bradycardia (Krayer and Acheson, 1946). The final proof of this concept is contained in the observation that the vasodilatation in the splanchnic area and the leg, which ensues on single intravenous injections of veratridine in the cat, is abolished by cooling the vagi to 8° C., thus blocking the *afferent* path of the reflex (Dawes, Mott, and Widdicombe, 1951). From general physiological principles it would indeed be surprising if a reflex mechanism which caused a profound bradycardia did not simultaneously cause vasodilatation ; the co-ordination of cardiac and vasomotor centres might be supposed to lead to such a response.

This argument can be used another way round. A drug which causes peripheral vasodilatation by a direct action on the central nervous system might be expected also to cause bradycardia. And this would appear to be true of veratridine, since Krayer, Wood, and Montes (1943) showed that injection of veratridine into the separately perfused central nervous system of the dog caused a considerable fall of heart rate. We may conclude therefore that there is *prima facie* evidence (though naturally this is not established for every veratrum alkaloid) that these substances may cause both a fall of blood pressure and of heart rate both through the Bezold reflex and by a central nervous action.

The experiments recorded above with cats show that under light chloralose anaesthesia the fall of blood pressure during an infusion of veratridine is almost entirely due to the Bezold reflex. Presumably the peripheral receptors are in this instance more sensitive to the alkaloid than the cells of the central nervous system. In dogs under chloralose anaesthesia the results were somewhat different, but even in this species there were two experiments which clearly led to the same conclusion. It may be observed that our experiments differed from those of Stutzman in two important respects. He used pentobarbitone (nembutal) anaesthesia, and it is well known both that the Bezold reflex is particularly sensitive to changes in the depth of anaesthesia and that it is much less easy to obtain clear evidence of reflex peripheral vasodilatation under barbiturates. Another possible reason for the difference in our results may be that we did not cut the vagi, but only cooled them. In this way we were able to provide a control observation when they were warmed again, a feature of the experiments which is important when using a drug which can only be injected or infused at relatively long intervals of time.

We cannot tell either from our own experiments or those of Stutzman *et al.* which of the two mechanisms, Bezold reflex or central action, is the more important in man. It may, however, be timely to recall that the reflex cardiovascular reaction first described by von Bezold and Hirt (1867) in rabbits on injection of crude extracts of veratrum alkaloids, has only within the last five years been localized with certainty to an action on receptors in the coronary circulation, that these hypothetical receptors have not yet been identified, that direct evidence for their existence has only been obtained as yet in the cat and the dog, and that there are some indications of a difference even between these two species.

Summary

Veriloid, a mixture of veratrum alkaloids in clinical use for producing a fall of blood pressure, does not materially differ in its mode of action from that of the pure veratrum alkaloids. It elicits the Bezold reflex and sensitizes or excites the pulmonary stretch receptors.

The fall of blood pressure and heart rate caused by a slow infusion of veratridine in cats is abolished by cooling the vagi. In some, but not all, dogs a similar result is obtained under chloralose anaesthesia. In view of this apparent species difference it would be unwise to draw any conclusions about the precise mode of action of these drugs in man. There is the possibility both of a peripheral Bezold reflex and of a central nervous action.

We are most grateful to Dr. R. Kauntze for a supply of veriloid, and to Professor O. Krayer for the veratridine. This work was done during the tenure by J. G. W. of a grant from the Medical Research Council.

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THE ACTION OF DRUGS ON ISOLATED HUMAN BRONCHIAL CHAINS

BY

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In 1937, Sollmann and Gilbert observed under a microscope thin slices cut from human lungs obtained *post mortem* and from human foetal lung, and found that histamine and acetylcholine constricted the bronchi whereas adrenaline dilated them.

Recently, Castillo and de Beer (1947) introduced the use of an isolated bronchial muscle preparation consisting of a chain of guinea-pig tracheal rings. We have applied the same principle to the study of drug action on human bronchi, using a chain of bronchial rings. This preparation gives reproducible results and is suitable for quantitative work, and it has been used to study the activity of several broncho-constrictor and bronchodilator drugs and the antagonism between mepyramine and histamine. A similar preparation has recently been described in a preliminary communication by Rosa (1951).

METHODS

Source of material.—Most of the work was performed on pieces of lung removed at operation from patients with carcinoma or bronchiectasis.* In lung carcinoma, the tumour is often relatively localized, and it is then possible to dissect normal bronchi from unaffected parts of the lung. In the specimens of bronchiectatic lung we have had, the disease was usually more widespread, but one or more normal bronchi could generally be found. The surgeon was sometimes able to give guidance on this point.

Post-mortem material is not easy to obtain fresh, but on occasion lungs were received from fatal accident cases within one or two hours of death. A further source of material is human foetus from therapeutic abortions; with this material preparations can be made from the trachea and primary bronchi.

No difference in response to drugs was observed between material from these various sources. Some preparations deliberately made from bronchiectatic bronchi appeared to respond normally to drugs. Preparations from the bronchial muscle of pigs suffering from various forms of pulmonary disease were investigated by Macht and Ting (1921); they did not describe any abnormal responses to drugs, although they found reduction or absence of sensitivity in preparations from severely diseased animals.

Muscle strips from primary bronchi.—On one occasion muscle strips dissected from the primary bronchus of a human lung were examined. They were found to respond to drugs, but the responses soon decreased in size and had ceased altogether two or three hours after the preparations had been set up.

* We are grateful to Professor R. S. Pilcher and to Dr. H. Herxheimer, of the Surgical Unit, University College Hospital, for making the material available to us.

Experimental procedure.—On removal from the patient the lobe or lung was placed in cold Ringer solution and transferred to the laboratory. Suitable bronchi were dissected out and cut into rings, which were tied in chains with loops of cotton. The chains were suspended in Ringer solution at 37° C. aerated with oxygen through a fine sintered glass plate. Responses were recorded with a light balsawood frontal writing lever on a lightly smoked kymograph drum rotating at 1 mm. per minute. The tension on the lever was 200 mg. and the magnification × 12. All sizes, from secondary bronchi (lumen diameter 6–8 mm.) to fifth order bronchi (diameter about 1 mm.), were found to be suitable. In general three to five rings were adequate to give sufficiently large recorded responses for quantitative work, since the smaller bronchi contain progressively less cartilage and are capable of a proportionately greater degree of contraction.

Drugs were added to the bath or occasionally introduced dissolved in the Ringer solution. When the response to a dose had reached a steady level, the bath fluid was replaced every five minutes with fresh pre-warmed and pre-aerated Ringer solution until the preparation had recovered its previous tonus level. An automatic apparatus, modified from that described by Schild (1947), was utilized for this purpose when several preparations were being used simultaneously. The Ringer solution used in some of the experiments was that described by Krebs and Henseleit (1932), aerated with $O_2 + 5\%$ CO_2. A similar solution with one-tenth of the concentration of sodium bicarbonate, and aerated with O_2 alone, was used in the remainder of the experiments. Both solutions had a *p*H lying between 7.1 and 7.4 under the experimental conditions, and there was no appreciable difference between the behaviour of preparations in them.

Under these conditions the chains maintained a steady baseline and gave repeated steady or graded responses to bronchoconstrictor or bronchodilator drugs for 10 to 15 hours. In contrast we have found that preparations maintained in Tyrode's fluid, aerated with oxygen at 37° C., become insensitive and cease to give responses within an hour or two. The bronchi still respond to drugs when used after preservation in Ringer solution at 4° C. for two or three days.

The sympathomimetics were compared with synthetic *l*-adrenaline *B.P.* (Burroughs Wellcome & Co.) made up as hydrochloride. The other drugs used were *dl*-noradrenaline hydrochloride (Winthrop Stanley), *dl-iso*propylnoradrenaline sulphate (Burroughs Wellcome), *l*-ephedrine hydrochloride *B.P.* (Burroughs Wellcome), and aminophylline (Burroughs Wellcome). Fresh dilutions of adrenaline, noradrenaline, and *iso*propylnoradrenaline were made up every two or three hours in distilled water containing ascorbic acid (10^{-5}).

Drug concentrations are given in terms of the active base. The bath volume in all the Figures was 50 c.c.

Results

Action of parasympathomimetic drugs

Acetylcholine added to the bath causes the chains to contract. A contraction of 50 per cent of the maximum is obtained with concentrations of the order of 10^{-6}. Fig. 1*a* shows that the effects were repeatable and that graded contractions could be obtained. The responses are readily reversed on changing the bath fluid. Fig. 1*b* shows reductions of the response to acetylcholine by atropine in concentrations 1 : 2,500,000,000 and 1 : 500,000,000.

Pilocarpine and carbachol also contract the chains. Pilocarpine has about four times the activity of acetylcholine ; carbachol is also more active than acetylcholine. The responses to these drugs take longer to reach a steady level than those due to acetylcholine ; they also take longer to reverse when the bath is washed out.

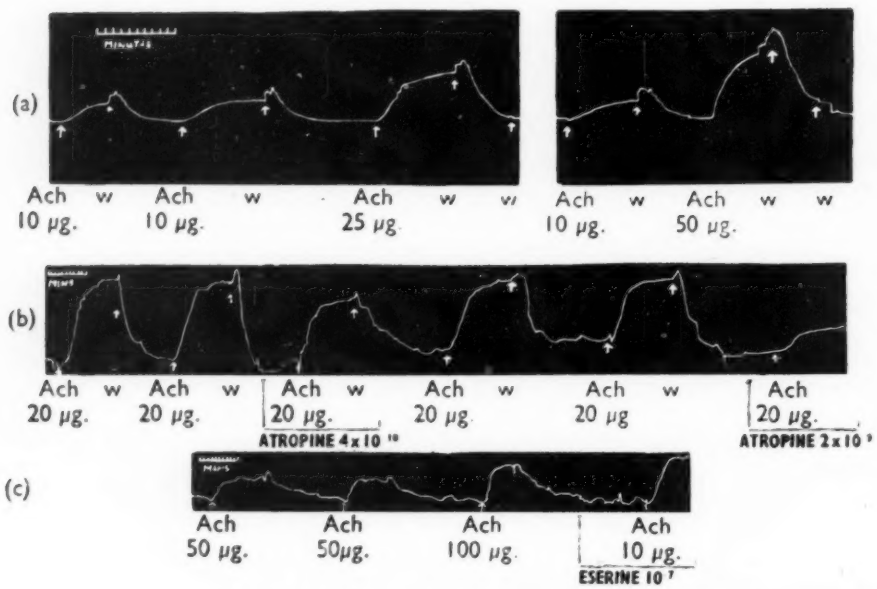

FIG. 1.—Human bronchial chains in 50 ml. modified Krebs–Henseleit solution. Intervals between injections 15–30 min. (*a*) Graded responses with acetylcholine (Ach). (*b*) Antagonism of acetylcholine by atropine. (*c*) Potentiation (> tenfold) of acetylcholine by eserine.

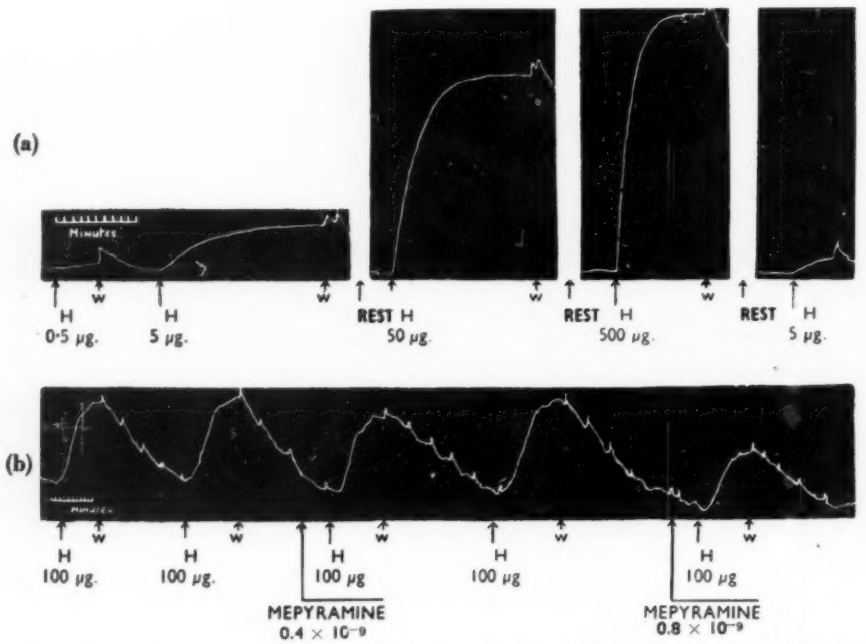

FIG. 2.—Human bronchial chain in 50 ml. bath. (*a*) Graded responses with histamine (H). (*b*) Antagonism of histamine by mepyramine.

Eserine in a concentration of 10^{-7} will potentiate the response to acetylcholine (Fig. 1*c*). Larger doses of eserine themselves cause slowly developing contractions which take a long time to reverse.

Action of histamine and mepyramine

Histamine contracts the chains when it is added to the bath. A 50 per cent contraction is obtained with concentrations of the order of 5×10^{-7}. (This figure is not directly comparable with that quoted for acetylcholine, since the maximum response obtained appears to differ for the two drugs.) Fig. 2*a* shows reversible graded responses. After several large doses of histamine, the response to a small dose is usually temporarily reduced. The active concentrations of histamine are of the same order as those which Castillo and de Beer (1947) found to contract the guinea-pig tracheal chain.

The response to histamine is reduced by mepyramine maleate in concentrations as low as 10^{-9}. This is illustrated in Fig. 2*b*.

pA_2.—pA_2 measurements (Schild, 1947) were made of this antagonism. This measurement expresses the negative logarithm of the molar concentration of an antagonist required to reduce the response to a double dose of active drug to the same height as the response to a single dose. The heights of contractions were measured from the baseline after the response had reached a steady level, generally after 10 or 15 minutes. The values were computed by interpolating graphically. A determination of pA_2 is shown in Fig. 3. The values obtained in preparations from three different lungs for periods of antagonist contact of 2, 10, and 30 minutes are shown in Table I, and compared with the values obtained by Schild (1947) and Reuse (1948) on guinea-pig ileum. The values on the human bronchi are in close agreement with those on the guinea-pig gut, except that the time required for the antagonist to reach equilibrium is longer with the bronchi.

Bronchoconstrictor action of mepyramine.—In higher concentrations, 10^{-5} and greater, mepyramine itself has a constrictor action. The response is reversible and repeatable and graded doses give graded responses, though the slope of the dose-response curve is less with mepyramine contractions than it is with contractions due to histamine (Fig. 4).

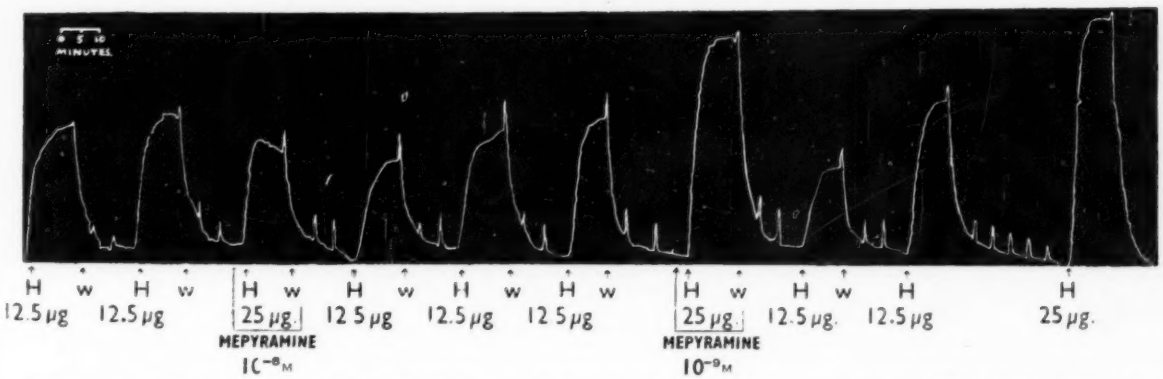
0 5 10
MINUTES
H w H w H w H w H w H w H w H w H w H
12.5 µg 12.5 µg 25 µg. 12.5 µg 12.5 µg 12.5 µg 25 µg. 12.5 µg 12.5 µg 25 µg.
MEPYRAMINE
10^{-8}M
MEPYRAMINE
10^{-9}M

FIG. 3.—Human bronchial chain in 50 ml. bath. Determination of pA_2 for mepyramine-histamine. The value determined from this experiment was 9.27.

TABLE I

pA_2 MEPYRAMINE-HISTAMINE ON GUINEA-PIG ILEUM AND HUMAN BRONCHIAL CHAINS

	pA_2 values for different times of antagonist contact*			
	2 min.	10 min.	14 or 15 min.	30 min.
Guinea-pig terminal ileum { Schild (1947) ..	8.71		9.46	
Guinea-pig terminal ileum { Reuse (1948) ..			9.32	
Human bronchial chain ..	8.27	8.75		9.26, 9.27

* The time of antagonist contact is the time between the addition of mepyramine to the bath and the dose of histamine.

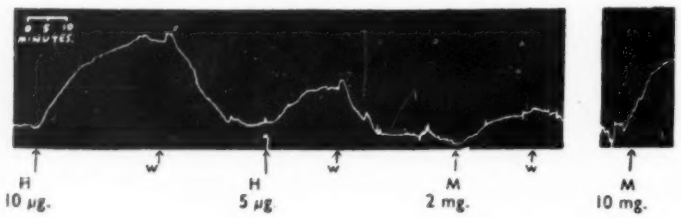

FIG. 4.—Human bronchial chain in 50 ml. bath. Bronchoconstrictor action of mepyramine (M). H = histamine.

Action of sympathomimetic compounds and aminophylline

The preparation has a good spontaneous tonus level, and sympathomimetic drugs such as *iso*propylnoradrenaline (10^{-8}), adrenaline (10^{-8}), noradrenaline (10^{-6}), and ephedrine (10^{-5}) relax the chains. Aminophylline (10^{-5}) also produces relaxation. Responses to *iso*propylnoradrenaline, adrenaline, or noradrenaline take from 10 to 25 minutes to reach a steady level, and recovery on washing out the bath takes from 20 to 40 minutes. Responses to aminophylline and ephedrine generally take longer to develop than equivalent responses to adrenaline, and the recovery from a fairly large dose of ephedrine may take as long as one hour. However, the stability of the preparation is such that it is often possible to obtain regular responses to these compounds over periods as long as 12 hours. Satisfactory graded responses are obtainable, and it is possible to compare the relative potency of bronchodilator drugs directly.

Comparative assay of bronchodilator drugs.—For this purpose we have used a four-point technique, with two doses of each drug. The order of doses within a group of four responses was randomly chosen. Fig. 5 shows a comparison of ephedrine with adrenaline. The responses were measured from the baseline to the level at which they became steady and the relative potencies computed graphically. The preparations were left at least one hour before any doses were given. Allowing for one or two preliminary doses, and doses to confirm the steadiness of the response and determine the maximum response at the end, a single comparison consisting of a group of four responses took between six and ten hours to make. Table II shows the results of comparisons of the activity of bronchodilator drugs with adrenaline obtained in different preparations at different times. Four comparisons were made with each drug, and the final result is expressed as the geometric mean of the activity ratios. As might be expected the activity ratio for the same

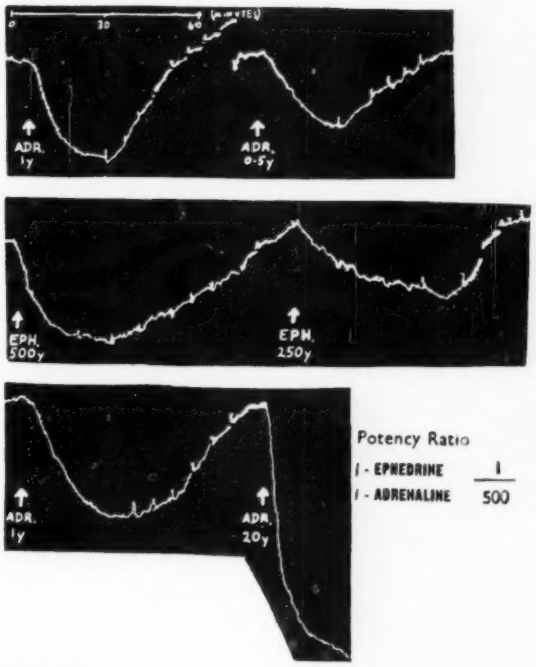

FIG. 5.—Human bronchial chain in 50 ml. bath. "Four-point" comparison of bronchodilator activity of adrenaline and ephedrine, giving a potency ratio of 500. The bath was washed out at the lowest point of each tracing.

TABLE II

ACTIVITY OF BRONCHODILATORS

	Relative potency *l*-adrenaline = 1,000	Geometric mean
*dl-iso*propylnoradrenaline	5,600 5,200 4,200 7,200	5,400
dl-noradrenaline	67 61 62 15	44
l-ephedrine	1.1 0.4 2.1 3.6	1.4
aminophylline	0.51 1.23 0.17 0.76	0.53

pair of drugs varies from experiment to experiment, but this variation is small compared with the variation between different drug pairs. The relative potencies are expressed in terms of the active base for the sympathomimetic amines and in terms of the complex salt for aminophylline.

Discussion

The evaluation of the action of drugs on the bronchi in man is usually based on vital capacity measurements. The interpretation of the changes in vital capacity caused by drug administration is difficult, since these measurements give no indication of the site of action or mode of action of the drug, which may act peripherally, centrally, or reflexly. Even if the drug is given by inhalation there is no guarantee that the effects are purely peripheral, since systemic effects are often observed. Also, the action of the drug may be to stimulate secretion of mucus, or cause oedema of the mucous membrane, thereby producing a reduction of vital capacity; it is not possible to determine what proportion of the effect observed is due to contraction of the bronchial musculature. A further complication lies in the mode of administration. Intravenous administration, which gives the most strictly comparable results, is frequently unsuitable, and when drugs are administered by inhalation their concentrations at the site of the action are not known.

There are additional difficulties with bronchodilator drugs, since in general it is necessary to induce some degree of bronchospasm with constrictor drugs and then to test the effect of the bronchodilators in relaxing this spasm. The effects recorded in this type of experiment may not indicate a general bronchodilator activity but merely demonstrate some specific antagonism. An alternative is to use emphysematous patients with some degree of pathological bronchial obstruction.

An isolated preparation of the type we have described avoids most of these difficulties and provides a direct measure of the activity of drugs on the smooth muscle of the bronchi. The chain preparation possesses the advantages that it records purely the circular component of responses and that it can be used for small bronchi. In addition it has a sufficiently high tonus level to permit direct examination of bronchodilator actions.

In general, the sensitivity of the preparation to drugs seems to resemble closely that of the guinea-pig tracheal chain. Castillo and de Beer (1947) found the guinea-pig tracheal chain to be contracted by histamine and acetylcholine and relaxed by adrenaline and aminophylline. The effective concentrations were of the same order as those required in the human bronchial preparation.

Sympathomimetic amines and aminophylline

Our results show that the sympathomimetic amines relax the resting tone of isolated human bronchial muscle and that comparisons of activity can be achieved without previously contracting the muscle with drugs. The comparative potencies of the amines we have tested show no marked differences from the results of previous workers who examined the activity of sympathomimetics on bronchial muscle that had previously been constricted by histamine, pilocarpine, mecholyl, or barium. Table III shows the results obtained on the human bronchial chain compared with values taken from the literature, and representative ranges of the therapeutic doses employed clinically in asthma.

A drug which will relax the unstimulated human bronchial chain is not only a true bronchodilator, but also can be expected to produce a bronchodilatation in the intact human subject. Comparisons of the results on human bronchial muscle with the therapeutic doses used clinically in asthma suggest that the therapeutic activities of the sympathomimetic amines tested and of aminophylline are largely dependent

TABLE III

EQUIACTIVE DOSES OF BRONCHODILATOR DRUGS

Author:	Present work	Tainter *et al.* (1934) Luduena (1942)	Konzett (1940)	Bresnick *et al.* (1949)	Therapeutic doses	
Preparation:	Human bronchial chain	Perfused guinea-pig lung	Chloralosed dog	Vital capacity (asthmatic human subjects)	Intra-venous	Sub-cutaneous
Constrictor agent:	None	Histamine, pilocarpine, barium	Pilocarpine	Histamine mecholyl		
*dl-iso*propylnor-adrenaline ..	0.19		0.1	0.5		
l-adrenaline ..	1	1	1	1	1 (dose= 0.1 mg.)	1 (dose= 0.5 mg.)
dl-noradrenaline	23	7				
l-ephedrine ..	710	335				30–120 (dose= 15–60 mg.)
aminophylline	1,900	1,250–1,870			2,500–5,000 (dose= 0.25–0.5 g.)	

on their activity in directly relaxing bronchial muscle. All these drugs, including ephedrine, actively dilate the isolated human bronchial muscle and produce constant reversible effects without tachyphylaxis. It is therefore unlikely that their activity is due to potentiation of adrenaline ; their action is probably direct.

The ratio of activity of adrenaline base and aminophylline in the bronchial chain is 1,900 ; if the ratios of their therapeutic activities were the same 0.5 g. aminophylline intravenously should produce the same effect as 0.25 mg. adrenaline intravenously. This may well correspond to their relative clinical activities and explain why aminophylline is sometimes effective in asthma when adrenaline fails (Sollmann, 1948) ; an intravenous dose of 0.5 g. aminophylline can be safely administered, whereas an intravenous dose of 0.25 mg. adrenaline cannot be given without producing severe circulatory effects.

The ratio of activity of adrenaline and ephedrine on isolated human bronchi is 710. The ratio of the customary therapeutic doses is 100 or less (subcutaneously 0.5 mg. adrenaline or 15–60 mg. ephedrine), but the true equitherapeutic ratio in asthma is almost certainly higher (Herxheimer, 1946).

Mepyramine

It is interesting to note that, although guinea-pig ileum is fifty or a hundred times more sensitive to histamine than human bronchi, the pA_2 value for mepyramine against histamine is similar in the two preparations.

The bronchoconstrictor action we have observed with higher concentrations of mepyramine is reminiscent of the contractile action of antergan and mepyramine

on the guinea-pig uterus described by Bovet and Walthert (1944). This effect may be of clinical importance, since, although, as these experiments show, the concentrations of mepyramine required to antagonize histamine added to the bath are extremely low, the concentrations which are required to reduce anaphylactic or allergic reactions are very much higher (Schild, Hawkins, Mongar, and Herxheimer, 1951).

Summary

1. A preparation for the study of drug action on human bronchi, consisting of a chain of bronchial rings, is described.

2. Acetylcholine, pilocarpine, and carbachol contract the preparation. The action of acetylcholine is antagonized by atropine and potentiated by eserine.

3. Histamine contracts the preparation, and its action is antagonized by mepyramine. pA_2 values determined for this antagonism indicate that the histamine response is reduced by mepyramine in the same concentrations as are effective on guinea-pig ileum. Higher concentrations of mepyramine have a bronchoconstrictor action.

4. Adrenaline, noradrenaline, *iso*propylnoradrenaline, and aminophylline relax the human bronchial chain. Their relative potencies have been determined.

5. The results agree closely with those found by other workers using animal preparations.

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Brit. J. Pharmacol. (1951), **6**, 691.

DECURARIZATION BY DECAMETHONIUM

BY

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Paton and Zaimis (1949) have drawn attention to the curious relationship between decamethonium and *d*-tubocurarine. They found that previous administration of a small dose of tubocurarine rendered decamethonium less effective as a neuromuscular blocking agent ; on the other hand, decamethonium given before tubocurarine did not protect against block by the alkaloid. From these observations it has been inferred that decamethonium does not antagonize the action of *d*-tubocurarine (Castillo, Phillips, and de Beer, 1949 ; Macfarlane, Unna, Pelikan, Cazort, Sandove, and Nelson, 1950 ; Winter and Lehman, 1950). The experiments here reported show that this is only partly true, for in suitable doses decamethonium restores neuromuscular transmission after block by tubocurarine.

METHODS

Cats, decerebrate or anaesthetized with chloralose, were used. Tension was recorded from tibialis anterior excited through its nerve by supramaximal shocks once every 10 seconds. For close arterial injection the muscle was prepared as described by Brown (1938). End-plate potentials were recorded from the gracilis muscle (Brown and Burns, 1949) by means of fine platinum electrodes about 5 mm. apart and a condenser coupled amplifier whose time constant was 0.41 second. The infusion apparatus described by Burn and Dale (1924) proved suitable for intravenous administration of *d*-tubocurarine chloride. It consists of a burette whose delivery is controlled by the flow of oil through a capillary tube. For initial curarization a positive pressure was created in the oil reservoir until the desired degree of neuromuscular block was attained. Curarization was then maintained by suitably adjusting the height of the reservoir. The rate of infusion necessary to maintain block varied greatly between preparations, and the range, in our experience, extended from 0.25 to 1.1 mg./kg./hr.

RESULTS

The experiment illustrated in Fig. 1 shows the effect of close arterial injection of decamethonium into a preparation which had received enough tubocurarine to reduce the twitch tension to 10 per cent of its initial value. As little as 0.8 μg. decamethonium iodide produced a distinct decurarization which lasted 20 minutes. With twice this amount restoration of transmission was more complete, and the twitch tension reached 80 per cent of its initial value. With doses in this range the first few twitches after the injection grew, their peaks forming an upward concave curve. When 3.2 μg. decamethonium was given a new phenomenon made its appearance. The response to the first stimulus after the injection was the largest ; after this the twitch tension decreased for three minutes, but then waxed and waned again. With

* Sharpey Scholar.

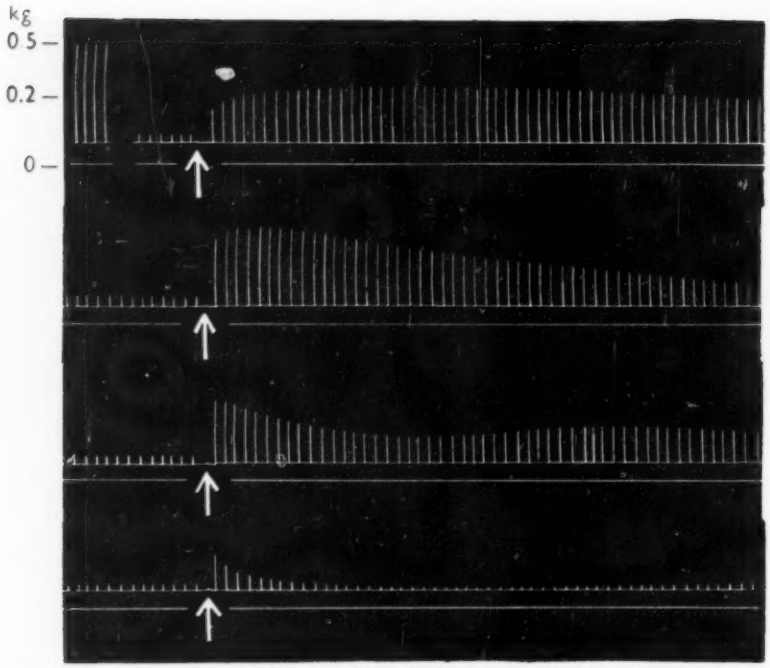

FIG. 1.—Cat, 2.5 kg. Chloralose. Contractions of tibialis anterior in response to maximal stimuli to nerve every ten seconds. Preparation partly curarized. At arrows, from above downwards, close arterial injection of 0.8, 1.6, 3.2, 6.4 μg. decamethonium iodide. Top left: twitches of untreated muscle.

yet larger doses of decamethonium restoration of transmission was slight and transient, and rapidly gave way to a renewed block.

Decurarization can similarly be observed on intravenous administration of decamethononium in doses which, given alone, produce complete neuromuscular block. In Fig. 2*b* the result of injection of 60 μg./kg. of decamethonium iodide into the saphenous vein is shown. The twitch tension increased gradually to about 80 per cent of its value at the beginning of the experiment and remained there for some minutes before declining again. In the same preparation, at somewhat deeper curarization, the effect of five similar doses, given in close succession, was tested (Fig. 2*c*). It can be seen that the first two injections progressively increased the twitch tension, but that subsequent doses did so only transiently and, in the main, had the opposite effect. In this experiment we thus traversed the whole cycle, from block by *d*-tubocurarine to block by decamethonium. The fact that decamethonium, when enough is given, will block transmission even in the presence of tubocurarine is, no doubt, the reason why its decurarizing property has been overlooked so far, and it probably underlies yet another curious feature of its action. Under our experimental conditions we have never seen complete restoration of transmission, as judged by the response to a single shock, and it seems likely that decamethonium, in the concentration required to decurarize the most resistant neuromuscular junctions, blocks those junctions most sensitive to it.

It is known that decamethonium is a weak inhibitor of cholinesterase (Barlow and Ing, 1948; Paton and Zaimis, 1949). To test whether the decurarization

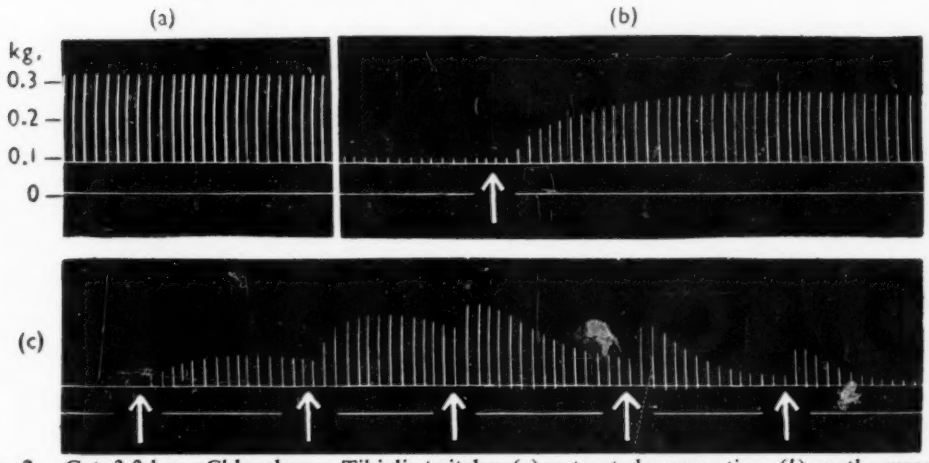

FIG. 2.—Cat, 3.3 kg. Chloralose. Tibialis twitches (*a*) untreated preparation, (*b*) partly curarized; at arrow intravenous injection of 200 μg. decamethonium iodide; (*c*) 2 hr. later; at each arrow intravenous injection of 200 μg. decamethonium iodide.

described here can be attributed to this property of the compound the following experiment was made. A heavily curarized preparation was treated, by retrograde injection into the opposite femoral artery, with 250 μg. physostigmine sulphate. The resulting decurarization is shown in Fig. 3*a*. More tubocurarine was then given until complete block was re-established and the process then repeated four times. The fifth dose of physostigmine no longer restored transmission, but subsequent doses of decamethonium decurarized effectively (Fig. 3*b*). Hence the two compounds do not act in the same manner.

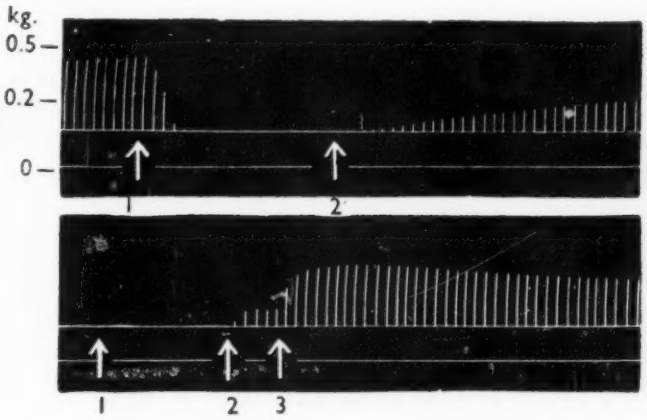

FIG. 3.—Cat, 3.0 kg. Chloralose. Contractions of tibialis. (*a*) At arrow 1, intravenous infusion of *d*-tubocurarine chloride; at arrow 2, "distant" arterial injection of 250 μg. physostigmine sulphate; (*b*) 30 min. later, after treatment described in text, "distant" arterial injections of, at arrow 1, 250 μg. physostigmine sulphate, at arrow 2, 50 μg., at arrow 3, 100 μg. decamethonium iodide.

In order to obtain better insight into these phenomena we studied the electrical responses in gracilis. The muscle was stimulated through its motor nerve at 10-second intervals either by a single supramaximal shock or by a pair of shocks 1.2 msec. apart. Curarization was taken to the stage at which both forms of stimulation failed to evoke muscle action potentials (Fig. 4*a, b*): that is a good deal further than in most of the preceding experiments. Continuous recording of endplate potentials was then made while decamethonium was injected at an even rate into a

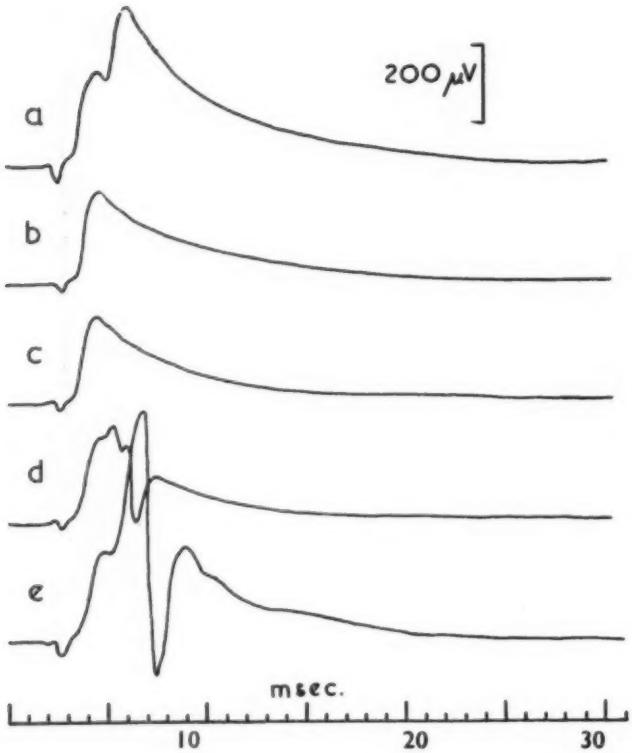

FIG. 4.—Cat, 3.2 kg. Chloralose. Curarized. Endplate potentials recorded from gracilis. (*a*) Response to two supramaximal stimuli to nerve 1.2 msec. apart; (*b–e*) responses to single supramaximal stimuli to nerve; (*a*) and (*b*) before decamethonium; (*c–e*) during slow infusion of decamethonium, 0.35 mg. per min.; (*c*) 3 min. 20 sec., (*d*) 3 min. 30 sec., (*e*) 3 min. 50 sec. after commencement of infusion.

vein. This procedure was adopted in order to make sure that an effective concentration was reached slowly so that we might observe any change in the endplate potentials before their form became distorted by spikes. In all 1.4 mg. decamethonium iodide was given in the course of 4 minutes. Records (Fig. 4*c, d, e*) taken 3 minutes 20 seconds, 3 minutes 30 seconds, and 3 minutes 50 seconds after commencement of injection demonstrate that muscle action potentials appear without any detectable increase in the height of the endplate potentials or prolongation of their time course.

DISCUSSION

Paton and Zaimis (1949) have shown that a dose of decamethonium which, given alone, produces neuromuscular block is without effect when given to a preparation which has just recovered from a small dose of *d*-tubocurarine. We have used enough tubocurarine to cause almost complete block and found that under those conditions the most striking action of decamethonium is to restore transmission.

Decurarization by decamethonium takes place without change in the peak voltage or time course of the endplate potential, indicating that both the intensity of the transmitter action and the activity of the cholinesterase remain unaltered. The site of action must therefore be more peripheral. This agrees with what is already known about this compound. Decamethonium blocks neuromuscular transmission by depolarizing the motor endplate region (Burns, Paton, and Dias, 1949), and this property provides an explanation for its anticurare activity as well, if it is assumed that in the curarized preparation it exerts a slight depolarization only.

The amount of further depolarization required for initiation of endplate potentials would then be reduced. Such an additive effect accounts satisfactorily for the observation that an endplate potential which previously failed to reach threshold will, after decamethonium, set up action potentials without any detectable increase in its size.

When very large doses of decamethonium were given restoration of transmission was poor. There is thus an optimal concentration for decurarization beyond which a stage is no doubt reached where an increasing number of fibres becomes blocked by too much depolarization. Moreover, the less deep the level of curarization the more readily does the blocking action of decamethonium become apparent.

Recently the pharmacology of a series of phenolic quaternary ammonium compounds has been studied in some detail (Riker, Wescoe, and Brothers, 1949 ; Randall, 1950 ; Riker and Wescoe, 1950). These compounds share with decamethonium the property of being both neuromuscular blocking and anticurare agents, and it seems likely that they act in a similar manner. Their anticurare activity has received special attention and has been tested on anaesthetized surgical patients (Artusio, Riker, and Wescoe, 1950) and on conscious volunteers (MacFarlane, Pelikan, and Unna, 1950). The last group of authors have also reported that these compounds were effective in alleviating muscular weakness in a myasthenic patient. If a case for the use of decurarizing agents which act by virtue of their direct effect on the motor endplates can in fact be made, then decamethonium should be the compound of choice, since it is free from the undesirable side-effects on the heart and circulation which the phenolic quaternary ammonium compounds display in varying degrees. Our experiments point, however, to an inherent difficulty in the use of anticurare agents of this type. Their action is of short duration, compared with anticholinesterase drugs, yet any attempt to prolong it by increasing the dose entails the risk of reaching concentrations in which these agents will themselves block transmission.

Summary

In suitable doses decamethonium restores neuromuscular transmission after block by *d*-tubocurarine. This decurarization is not due to inhibition of cholinesterase by decamethonium. Transmitted responses appear without detectable increase in size or prolongation in time course of the endplate potential. These experiments emphasize the inverse relationship between the two neuromuscular blocking agents.

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A METHOD FOR TESTING MIOTICS ON THE MOUSE PUPIL

BY

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At present there is no good method available for testing the activity of substances which constrict the pupil. Aeschlimann and Reinert (1931), in their investigation of analogues of physostigmine, tested substances by instillation into the eye of the cat, comparing the pupil of the eye with that of the other eye. Fellows and Livingston (1940) made similar observations on furfurylammonium iodides using rabbits.

Pulewka (1932) described a quantitative method for testing mydriatics on the pupil of the mouse, in which he determined the mean effect on the diameter of the pupil in groups of mice. This method was used extensively by Ing, Dawes, and Wajda (1945) in the work which led to the introduction of lachesine as a mydriatic. It seemed possible that a method for testing miotics might be based on their ability to antagonize the action of atropine when Pulewka's procedure was followed. If different groups of mice were injected with a given dose of atropine so as to produce a moderate mydriasis, then miotic substances might be compared by finding the dose required to reduce the size of the pupil to the same extent.

METHOD

In Pulewka's method, as it has been used in this laboratory, groups of five mice are taken. They are not kept without food beforehand. The pupil of each mouse is examined under a binocular microscope ($\times$ 10) which has a scale in the eyepiece. A small lamp is attached to the microscope so as to shine on the object, and the diameter of the pupil when thus illuminated is measured on the scale. The mean figure for the pupil of normal mice is about two divisions (7.7 divisions = 1 mm.). If mice are given an intraperitoneal injection of atropine sulphate and are examined about 15 min. later, the mean diameter of the pupil increases in linear proportion to the log-dose. Thus 2 μg. atropine increases the mean size to about 4 divisions, 4 μg. increases it to about 8 divisions, and 8 μg. increases it to about 14 divisions (Ing, Dawes, and Wajda, 1945).

In the present investigation groups of mice were taken, in any experiment mice of the same range in weight being used. Mice from 15–20 g. are more sensitive to atropine than heavier ones. The solution of the miotic was mixed with a solution of atropine sulphate, and a volume of the mixture was injected intraperitoneally which contained 4 μg. atropine sulphate and a certain weight of the miotic in 0.2 ml. The pupil of the mouse was examined after 15 min.

Substances investigated.—The substances tested were in the first place compounds prepared here by Dr. H. R. Ing and Mr. D. P. H. Tudor Williams, namely:

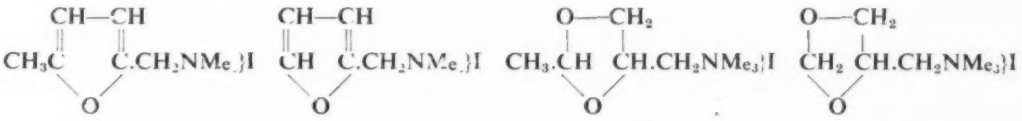
(*a*) 5-Methyl-furfuryltrimethylammonium iodide (I), referred to as the methyl-furfuryl compound.

(*b*) Furfuryltrimethyl ammonium iodide (II), referred to as the furfuryl compound; this substance is marketed as Furmethide (Smith, Kline and French); it was previously examined by Fellows and Livingston (1940).

Through the kindness of Dr. A. J. Ewins, F.R.S., of Messrs. May & Baker, there were available:

(*c*) Compound 2268F (Fourneau, Bovet, Bovet, and Montezin, 1944), the acetal of 2 : 3-dihydroxypropyltrimethylammonium iodide (III), referred to as the acetal compound; and

(*d*) Compound 2249F (Fourneau *et al.*, 1944), the corresponding formal derivative (IV), referred to as the formal compound.



I II III IV

The following substances were also examined:

(*e*) Carbamylcholine (carbachol).

(*f*) Eserine sulphate.

(*g*) Neostigmine methylsulphate.

RESULTS

Carrying out the test.—In preliminary tests, groups of five mice were injected with varying doses of each miotic in order to determine the range of activity. A dose which reduced the effect of atropine to about half was selected together with doses smaller and larger. The results of one experiment with the methyl-furfuryl compound are given in Table I.

The final observations with the different compounds are recorded in Table II. When the mean results were plotted against the log dose as in Fig. 1, a linear relation between dose and pupil diameter was obtained. The lines for eserine, the methyl-

TABLE I

Diameter of pupil (in scale divisions) after injection with mixture containing 4 μg. atropine and the stated amount of the methyl-furfuryl compound

Group 1 5 μg.	Group 2 10 μg.	Group 3 20 μg.	Group 4 40 μg.
4.0	7.0	2.5	1.0
7.5	2.5	5.0	2.5
4.0	4.0	6.0	1.5
3.5	3.5	2.0	2.0
5.5	3.5	2.0	4.0
5.0	4.5	4.5	2.5
2.5	3.5	2.0	2.5
5.0	2.5	2.0	2.0
8.5	5.0	4.0	4.0
4.5	4.5	5.0	1.5
Mean 5.0	4.05	3.5	2.35

TABLE II

Mean results with different doses, each dose being given together with 4 μg. atropine; size of pupil in scale divisions

Compound	No. of mice	Dose μg.	Size of pupil	Dose μg.	Size of pupil	Dose μg.	Size of pupil	Dose μg.	Size of pupil
Methyl-furfuryl ..	20	5	5.5	10	4.4	20	3.6	40	2.7
Furfuryl	30	40	5.9	80	4.5	160	4.05	—	—
Acetal	30	5	6.15	10	5.1	20	3.55	40	2.4
Formal	40	40	6.0	80	5.7	160	5.4	—	—
Carbachol	30	10	5.75	20	4.3	40	3.7	80	2.45
Eserine	25	—	—	2.5	3.6	5.0	2.85	10	2.0
Neostigmine	20	0.625	4.2	1.25	2.8	2.5	1.3	—	—

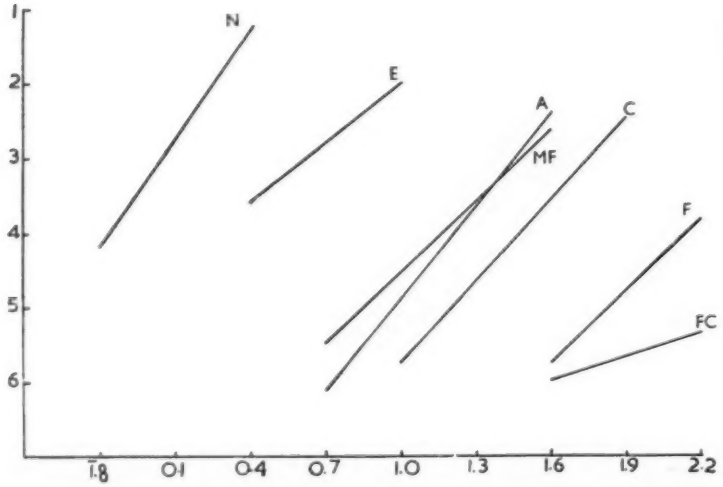


FIG. 1.—To show the relation between log dose of the miotic (abscissae) and the effect measured in scale divisions (ordinates). Since each dose is given together with a standard dose of atropine, the greater the dose of miotic the smaller the pupil becomes. Hence the ordinate is marked in descending order of magnitude. N = neostigmine, E = eserine, A = acetal compound, MF = methyl-furfuryl compound, C = carbamylcholine, F = furfuryl compound, and FC = formal compound.

furfuryl compound, the acetal compound, carbachol, and the furfuryl compound were approximately parallel. The line for neostigmine was steeper than the others, and the line for the formal compound was less steep than the others.

Neostigmine appears as the most potent miotic, and eserine is the next. The methyl-furfuryl compound and the acetal compound are again weaker and approximately equal, being stronger than carbachol, which is in turn stronger than the furfuryl compound. The formal compound shows only traces of activity.

Since the slope of the line for eserine is very nearly parallel to that of four of the other compounds, their activity has been expressed in relation to that of eserine taken as =100. The figures are given in Table III, in which an average figure for neostigmine is also included.

DISCUSSION

The evidence shows that substances with a muscarine-like action are not such strong miotics as substances which are powerful inhibitors of cholinesterase. It will be seen from Table III that neostigmine and eserine are far stronger than carbachol or the methyl-furfuryl compound or the acetal compound. Neostigmine, being more

TABLE III

RELATIVE MIOTIC ACTIVITY

Neostigmine	350
Eserine	100
Methyl-furfuryl compound	13.5
Acetal compound	13.5
Carbachol	5.0
Furfuryl compound	1.0
Formal compound	very weak

stable than eserine, is a powerful miotic. Aeschlimann and Reinert (1931) found neostigmine weaker than eserine on the eye of the cat, but, in a clinical trial in acute and chronic glaucoma, Clarke (1939) found neostigmine to be better than eserine in that it did not deteriorate and had a stronger and more prolonged action. He did not, however, test the two substances in the same concentration. It is probable that eserine is absorbed more readily than neostigmine from the conjunctival sac, and therefore when these substances are given by instillation the greater potency of neostigmine observed in the present experiments is not apparent.

Additional evidence that the anticholinesterases are more potent miotics than the muscarine-like compounds is provided by the well-known activity of di-*iso*propyl-fluorophosphonate, which continues for 6–27 hr. on the human eye (Leopold and Comroe, 1946).

Summary

1. A method is described for measuring miotic activity on the pupil of the mouse in which the miotic is given by intraperitoneal injection, together with a standard dose of atropine. The miotic then antagonizes the dilator action of atropine to an extent which is proportional to the log dose of the miotic.

2. Anticholinesterases are more powerful as miotics than muscarine-like substances; thus eserine is 20 times as potent as carbamylcholine, and neostigmine is 3–4 times as potent as eserine.

3. In addition to carbachol, the following substances have been tested: (*a*) the Fourneau compound 2268, the acetal of dihydroxypropyltrimethylammonium iodide; (*b*) F2249, which is the corresponding formal; (*c*) furfuryltrimethyl-ammonium iodide (Furmethide); (*d*) 5-methyl-furfuryltrimethylammonium iodide.

I wish to acknowledge my indebtedness to Professor J. H. Burn for his guidance and helpful criticism throughout the course of this work.

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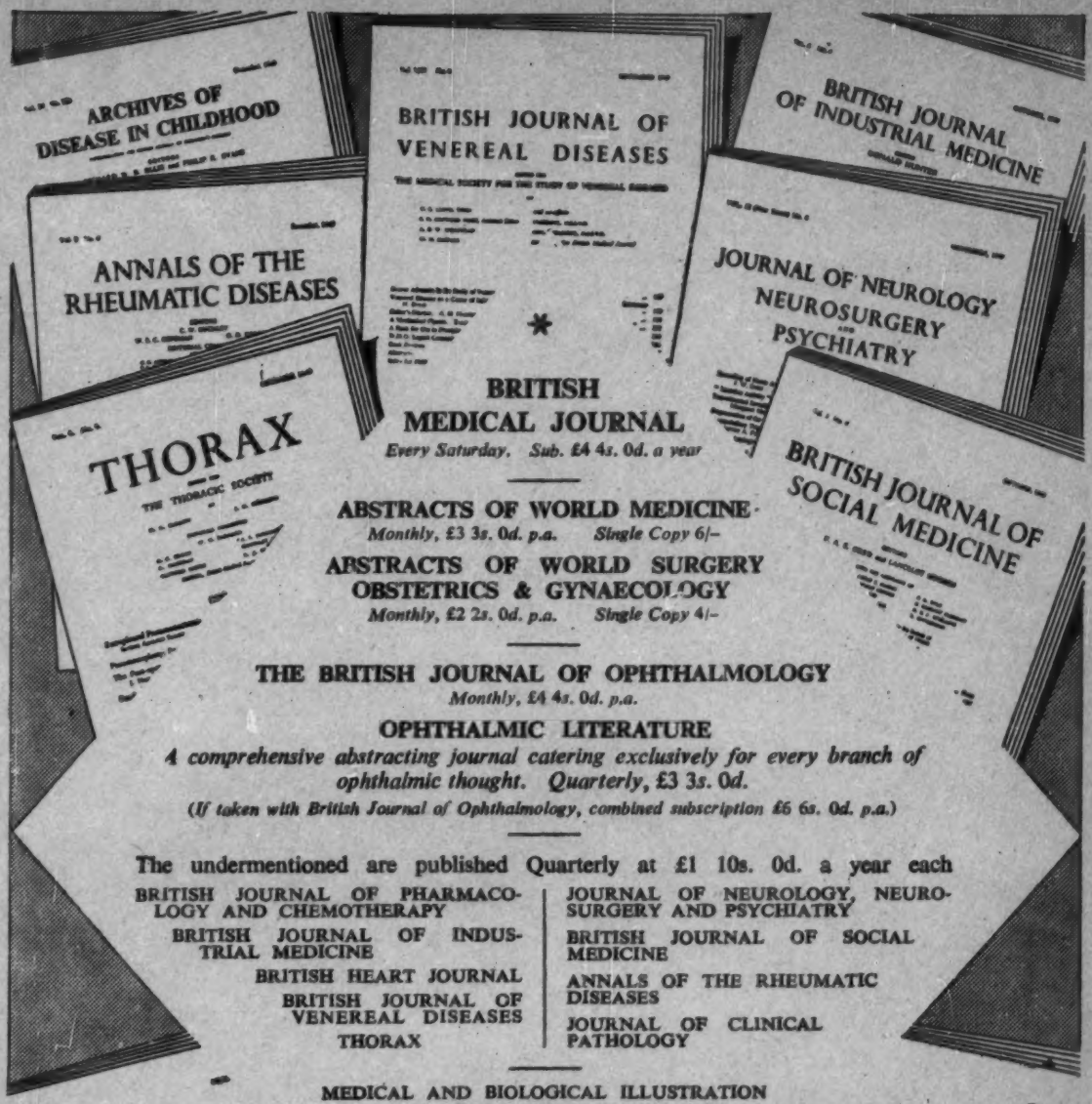
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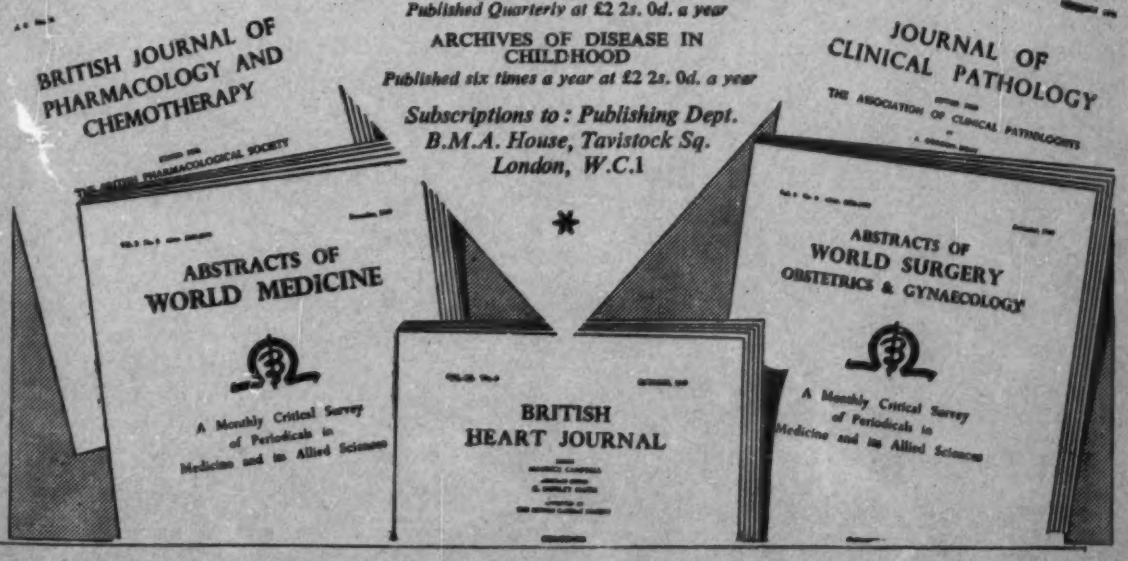
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